



Prevention of antibiotic residues

Influences of milking intervals and frequencies in automatic milking systems on excretion characteristics of different antibiotics in milk

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Prevention of antibiotic residues

Influences of milking intervals and frequencies in automatic milking systems on excretion characteristics of different antibiotics in milk

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Abstract

The prevention of antibiotic residues is an important aspect of milk quality. Limited information is available on the influence of milking frequencies deviating from regular milking of 2 times per day on the excretion of antibiotic residues in milk of treated cows.

As part of workpackage 5 within the European project "Implications of the Introduction of Automatic Milking on Dairy Farms" the excretion of antibiotic residues in milk of healthy cows was investigated. 4 different udder injectors containing 6 different antibiotics alone or in combination were tested. 3 groups of 5 cows were tested per drug. The experimental groups were milked three times per day (milking interval 8 hours) respectively 1.5 times per day (milking interval of 16 hours) and the excretion of antibiotic in cow composite milk was compared to a reference group milked 2 times per day (milking interval 10 and 14 hours).

Different approaches for determination of the withholding period were applied.

For three drugs tested the milking frequency had a significant influence on the excretion period: with increasing milking frequency the excretion period decreased. The differences were significant between groups milked 3 times and 1.5 times per day for all components of these three drugs. For one drug containing ampicillin and colistin the excretion period was not influenced by the milking frequency. Milk yield, somatic cell count and days of lactation had no significant influence on excretion of any antibiotic substance. The lactation number influenced the excretion time of cefquinome as well as of penicillin and dihydrostreptomycin in a combined drug, but for latter two drugs this effect was related to body weight..

Commercially available microbial inhibitor tests applied as screening methods for detection of antibiotic residues in milk detected penicillin G, ampicillin and nafcillin at concentrations below or at Maximum Residue Levels (MRL), whereas sensitivity for detection of cefquinome, dihydrostreptomycin and colistin was insufficient.

In a second part of the investigation the excretion time of antibiotic residues in milk of cows treated for clinical mastitis was determined. The results of that part will be presented in deliverable D12.

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1. Introduction

Recent investigations on milk quality during implementation of automatic milking systems mainly focused on parameters like somatic cell count (SCC), total bacterial count, freezing point and content of free fatty acids. Another important aspect of milk quality is the prevention of antibiotic residues in bulk tank milk. Severe penalties are applied in payment systems for ex-farm milk. Reasons are health protection for the consumer but also prevention of failures in technological processes in the dairy industry. An open question for automatic milking is whether a deviation from the regular milking scheme of two times per day influences the excretion characteristics of antibiotic residues in the milk of treated cows.

One of the main reasons for use of antibiotics in dairy cows is treatment of mastitis. Antibiotics are usually applied as udder injectors. Intramammary treatment is also most likely to be affected by different milking intervals, but only limited information is available mainly dealing with the influence of more frequent milking after treatment of clinical mastitis (Henschelchen and Walser, 1983). Irregular milking frequencies may occur in cows milked automatically. Therefore this study focuses on determination of excretion characteristics for different antibiotics after intramammary treatment of lactating cows milked with increased but also with reduced milking frequencies.

Antibiotics applied for mastitis therapy belong to several groups with different physico-chemical properties influencing the pharmacokinetics and thus the excretion via milk. Clinical mastitis affects the concentration of antibiotics in milk due to changes in pH of udder secretions and increased permeability of the blood milk barrier (Ziv, 1975).

In the first part of the investigation the excretion pattern was examined in healthy cows, because excretion characteristics in cows suffering from clinical mastitis are difficult to compare regarding severity of disease or mastitis pathogens involved.

Four different udder injector formulations containing 6 different antibiotic drugs were used for the experiments. For each drug 3 groups each consisting of 5 cows were milked with three different milking frequencies under experimental conditions. The time necessary for antibiotic residues in milk to fall short of maximum residue limits (MRL) fixed according to EU Regulation 2377/90/EEC were determined for the three groups. The excretion time in milk was compared to the indicated withholding period for the drugs applied. In addition, the sensitivity of commercially available screening tests for detection of the different antibiotic residues was determined.

In a second part of the investigation studies on the excretion of antibiotics were performed in cows with naturally occurring clinical mastitis. The results of this study will be presented in Deliverable D 12 and lead to recommendations for management of cows in automatic milking systems after treatment with antibiotics.

2. Literature review

2.1 Antibiotics in mastitis therapy

Physico-chemical properties

For treatment of mastitis it is important to reach high concentrations of antibiotics in udder tissue. The distribution of antibiotics is dependent on physico-chemical properties, because there is a pH difference between milk (pH 6.5-6.8) and blood serum (pH 7.4). Biological membranes like the blood udder barrier are only permeable for the non-ionized, lipid soluble part (Ziv, 1975). Weak acids like benzylpenicillin are mainly ionized if pH is higher than the pK-value. The concentration in blood is higher than in milk because with increasing pH a larger part of the substance is ionized. In contrast, weak bases like macrolides are mainly non-ionized at blood pH and can permeate the blood milk barrier. In milk the major part is ionized which leads to an enrichment of these substances in milk Ziv, 1975; Du Preez, 1988, Ehinger and Kietzmann, 1998).

With increasing pH during clinical mastitis weak acids reach higher concentrations in milk than in healthy udders due to restricted diffusion of non-ionized particles (Ziv 1975).

High degree of binding to udder tissue and/or milk proteins reduces the availability of antibiotics and effects the excretion after treatment (Du Preez, 1988).

Properties of the most important antibiotics applied for mastitis treatment are summarized in table 1.

Table 1: Chemical properties and milk to serum concentration ratio of antibiotics parenterally applied to cattle (adapted from ZIV, 1975)					
Antibiotic	Chemical nature	pK_a	Degree of lipid solubility	Milk to serum concentration ratio	
				normal milk	mastitic milk
b-lactam antibiotics					
<i>Penicillins</i>					
Benzylpenicillin	acid	2.7	moderate	0.12 - 0.20	0.25 – 0.32
Cloxacillin	acid	2.7	high	0.20 - 0.24	0.30 – 0.48
Ampicillin	acid	2.7, 7.2	high	0.22 - 0.30	0.28
<i>Cephalosporines</i>					
Cephaloridine	acid	3.4	moderate	0.24 - 0.28	
Cephaloglycin	acid	4.9	moderate	0.32 - 0.34	
Cefquinome	acid	2.5, 2.9	low		
Aminoglycosides					
Dihydrostreptomycin, Gentamicin, Neomycin, Kanamycin	base	9.0	low	0.20 - 0.80	0.35 – 0.70
Macrolides					
Erythromycin	base	8.8	high	3.00 - 4.00	1.80 – 2.60
Tylosin	base	7.1	high	3.60 - 5.40	1.00 – 1.40
Spiramycin	base	8.0	high	4.20 - 4.80	2.60 – 3.30
Lincosamides					
Lincomycin, Clindamycin	base	7.6	high	2.70 - 6.40	2.00 – 3.00
Polypeptides					
Polymyxin B, Colistin	base	10.0	low	0.20 - 0.40	0.38 – 0.70
Tetracyclines					
Tetracycline, Chlor- tetracycline, Dimethyl- chlortetracycline	amphoteric		low	0.92 - 1.28	

Only macrolides, lincosamides and some tetracyclines reach higher concentrations in milk of healthy cows than in blood serum (Table 1).

However, the selection of antibiotics for therapy is also dependent on the antibacterial properties of the drug.

Penicillins and macrolides are only effective against gram-positive bacteria except the broad spectrum penicillins like ampicillin or amoxicillin which also have an effect against gram-negative bacteria.

Cephalosporines have an extended spectrum compared to penicillins, but the spectrum is dependent on the type of cephalosporine. Aminoglycosides and tetracyclines are broad spectrum antibiotics and effective against gram-positive as well as gram-negative bacteria whereas polypeptides are mainly effective against gram-negatives.

Further aspects influencing the excretion of antibiotics via milk

The concentration of antibiotics in milk is also determined by the formulation of the drug. Sustained release-formulations where the molecules are incorporated into a particular matrix can achieve a certain concentration over a prolonged time period. Penicillin injected in oily suspensions is retained for longer periods than in oil-in-water emulsions. The shortest retention time is observed for aqueous solutions (Edwards, 1964). The injected volume does not influence the concentrations of penicillin in the udder.

In later stages of lactation irregular distribution of antibiotics in udder tissue was observed due to beginning involution accompanied by an increase of connective tissue (Edwards, 1964). A similar effect was found by inflammatory changes in cases of mastitis (Owens et al., 1990). Longer retention with higher concentrations of penicillin in the milk of quarters with chronic mastitis was found.

The findings reported on influence of milk yield on milk depletion time of different antibiotics were not consistent (Cannon et al., 1962; Whitem, 1999; Roncada et al., 2000). Probably only extreme low milk yields enhance the excretion period in milk. For penicillin excretion no relation was found with fat content of the milk and body weight (Cannon et al., 1962).

2.2 Usage of antibiotics in dairy cows

Limited information is available on the amount of antibiotics used in dairy cows in European countries. According to data from Denmark penicillins and cephalosporines alone or in combination with others accounted for about 95 % of intramammary treatments in 1998 and 1999 with a total of 500 kg active substance in 1999. These data are consistent with estimations from Germany indicating that for lactational therapy mainly β -lactam-antibiotics (cephalosporines and penicillins) are applied. Aminoglycosides (neomycin, gentamicin, dihydrostreptomycin or kanamycin), lincomycin and colistin are of minor importance.

Due to the European regulations a large number of veterinary drugs has lost approval at the end of June 2003, limiting the availability of certain antibiotics for dairy cow treatment.

2.3 Maximum Residue Limit (MRL)

Maximum Residue Limits for veterinary drugs in animal tissues and milk in the EU are based on the Acceptable Daily Intake (ADI) for the consumer calculated from the NOEL (No Observable Effect Level) and a safety factor. Safety considerations include toxicity, teratogenicity, mutagenicity, carcinogenicity and sensitizing potential for allergic reactions (immunotox-

icity). Also the NOEL regarding the human gut flora and starter cultures used in the dairy industry are taken into account. MRLs in milk are calculated on the basis of a consumption of 1.5 l milk per day.

Provisional MRLs with expiry date have been set for a number of old antibiotics for which the permanent approval is dependent on the provision of sufficient additional data (Council Regulation 2377/90/EWG ff.).

2.4 Withholding period

Withholding periods after treatment of animals should be appropriate to guarantee that concentrations of drug residues in edible tissues and milk do not exceed the MRL at the time of harvesting food of animal origin (milking or slaughter).

Withholding periods for milk should be determined by a uniform procedure according to the guidelines of the European Agency for the Evaluation of Medicinal Products (EMEA, 1998).

2.5 Selection of antibiotics for treatment trials

Based on the available information on usage of antibiotics in mastitis therapy in practice and the different pharmacokinetics of certain substances 4 different intramammary devices containing 6 antibiotic drugs alone or in combinations were selected: In the following the most important aspects are summarized for the antibiotics included in this study.

β -Lactam antibiotics

Penicillins are the most important group of antibiotics. The toxicity is low. Main excretion of penicillin is via urine, partly also via bile. Metabolism is of little importance in the elimination of penicillins.

Penicillins can produce allergic reactions in humans which is the most important side-effect in the use of penicillins. For inducing an allergic reaction a much higher oral dose is needed compared to parenteral administration (EMEA).

Starter cultures used in milk processing are very sensitive for penicillins. Concentrations below the MRL have significant influence on acid production by starter cultures (Suhren and Knappstein, 2002).

Procain-penicillin is a depot penicillin with sustained release of benzylpenicillin.

Ampicillin (AMP) is acid-stable with low protein binding.

Nafcillin (NAF) is a penicillinase-resistant penicillin. After intramammary application nafcillin is systemically resorbed, but in lactating cows the main part is excreted via milk.

For cefquinome (CEF), a cephalosporine, low systemic resorption occurs after intramammary application and excretion is mainly via milk. Metabolism is of low importance. NOEL for

Streptococcus thermophilus – a bacterium used in starter cultures - is 20 ng/ml (EMEA, 1995).

Dihydrostreptomycin (DHS)

DHS is a basic substance with high polarity. At physiological pH DHS is in ionized form with low penetration of biological membranes. Protein-binding is low. Excretion is dependent on formulation of the drug. There are no hints for metabolism of DHS in food-producing animals.

Colistin

Colistin is a polar substance with hydrophobic properties and highly irritating after intramuscular administration. Protein-binding is reduced with increased dose. Colistin is only active against gram-negative organisms, the gram-positive bacteria used as starter cultures in the dairy industry are not affected.

3. Methodology and study materials

3.1 Set up of experiments

3.1.1 Parameters for characterization of cows

The following parameters were used for selection and characterization of cows used in treatment trials:

- Number of lactation
- Days after calving
- Average milk yield per day during experimental period in kg
- Udder health status
- Body weight in kg (only determined in Nafpenzal[®]MC and Omnygram[®] trials)

Cows with comparable milk yield per day were used for treatment trials with different milking frequencies. Somatic cell count (SCC) in cow composite milk was below 100.000/ml in 3 weekly investigations before start of experiments and cows had not been treated with antimicrobials within a time period of 4 weeks before start of experiments.

3.1.2 Milking frequencies

For the treatment trials groups consisting of 5 (in two exceptions 4 resp. 6) cows were milked in a tandem milking parlour with three different milking frequencies per drug:

- 2 times per day at 4.30 a.m. and 2.30 p.m., milking intervals of 10 and 14 hours (reference group)
- 3 times per day at 4.00 a.m., 12.00 and 8.00 p.m., milking intervals of 8 hours, adjustment of cows to higher milking frequency 7 days before start of treatments
- 1.5 times per day at 4.00 a.m. and 8.00 p.m. every second day and at 12.00 every other day, milking intervals of 16 hours

Within each trial three milkings before the first treatment were used as anamnesis.

3.1.3 Sampling

Sampling was performed during the experimental period consisting of anamnesis, treatment period, withholding period for milk plus 2 additional days. If antibiotic residues in milk were detected more than two days after the end of the withholding period the experimental period was prolonged until samples of the respective cows reacted negative in screening tests during at least three milking times.

From every milking during the experimental period the following samples were taken:

- Quarter milk samples (at the beginning of milking):
 - 10 ml, preservation with potassium dichromate for determination of somatic cell count (SCC)
 - once during anamnesis without preservation for cyto-bacteriological investigation
- Cow composite milk:
 - 10 ml, preservation with potassium dichromate for determination of SCC
 - 250 ml, without preservation for determination of antibiotic residues

Samples were stored at 6 °C until analysis for 60 hours at maximum. Cow composite milk samples were stored at –20 °C for further investigations for 3 weeks at maximum. Samples for later re-examinations were preserved by lyophilization and stored at 6°C.

3.1.4 Drugs

For treatment trials the following commercially available udder injectors were used:

Table 2: Antibiotic drugs used for treatment trials

Drug	Cobactan® LC	Procain-Penicillin G 3 Mio.	Nafpenzal® MC	Omnygram®
Manufacturer	Hoechst Roussel Vet*, Unter-schleissheim, DE	WDT, Garbsen, DE	Intervet Int., Boxmeer, NL	Virbac S.A., Carros, France
Composition	88.8 mg Cefquinome(CEF)-sulfate = 75 mg CEF	3 g Procain-benzylpenicillin = 1898 mg Penicillin G (PEN)	300 000 I.U. Penicillin(PEN)-sodium = 180 mg PEN; 122 mg Nafcillin (NAF)-sodium = 100 mg NAF; 134 mg Dihydrostreptomycin(DHS)-sulfate = 100 mg DHS	100 mg Ampicillin (AMP)-trihydrate = 866 mg AMP; 250 000 I.U. Colistin (COL)-sulfate = 82.5 mg COL**
Total volume per injector	8 g	20 g	3 g	10 ml
Lot-No., Expiry date	01H033, 02/2003; 01H013, 09/2002 for repeated experiment with milking 2x per day	093090, 03/2002	not available	083B, 03/2004
Withholding time for milk	5 days	5 days	NL: 5 days UK: 3 days	6 days
MRL for milk	20 µg/kg	4 µg/kg	PEN: 4 µg/kg NAF: 30 µg/kg DHS: 200 µg/kg	AMP: 4 µg/kg COL: 50 µg/kg
Dosage	3 treatments, 4 quarters per cow (worst case), one injector per quarter per treatment was applied intracisternally			
Recommended treatment scheme	3 treatments during successive milking times	3 treatments with intervals of 24 hours	3 treatments with intervals of 24 hours	Treatment every 12 hours with at least one treatment after improvement of clinical symptoms
Recommended storage	below 30 °C	below 20 °C	below 20 °C	at 12°C – 15°C
Total amount of pure substance applied per cow (3x4 injectors)	900 mg CEF	22 776 mg PEN	2160 mg PEN 1200 mg NAF 1200 mg DHS	10392 mg AMP 990 mg COL

* today Intervet International; ** 1 IU = 0.033 µg colistin salt (Walter and Heilmeyer, 1975)

3.1.5 Treatment intervals

To apply the same quantity of antibiotic substance per time period the following treatment schemes were applied.

Table 3: Treatment scheme for Cobactan[®] LC and Omnygram[®]/2 times per day			
	Milking frequency		
	3 times per day	2 times per day	1.5 times per day
Anamnesis	day 1, 4.00 h	day 1, 14.30 h	day 1, 4.30 h
	day 1, 12.00 h	day 2, 4.30 h	day 1, 14.30 h
	day 1, 20.00 h	day 2, 14.30 h	day 2, 4.00 h
Treatment: 3 times per 24 hours	day 1, 20.00 h	day 2, 14.30 h	day 2, 4.00 h
	(day 1, 4.00 h)	day 3, 4.30 h	day 2, 12.00 h
	day 2, 12.00 h	day 3, 14.30 h	day 3, 4.00 h
	day 2, 20.00 h		(day 3, 20.00 h)
Milking times in brackets: no treatment			

Table 4: Treatment scheme for Penicillin G, Nafpenzal[®] MC and Omnygram[®]/3/ 1.5 times per day			
	Milking frequency		
	3 times per day	2 times per day	1.5 times per day
Anamnesis	day 1, 4.00 h	day 1, 14.30 h	day 1, 14.30 h
	day 1, 12.00 h	day 2, 4.30 h	day 2, 4.30 h
	day 1, 20.00 h	day 2, 14.30 h	day 2, 20.00 h
Treatment: 3 times per 48 hours	day 1, 20.00 h	day 2, 14.30 h	day 2, 20.00 h
	(day 2, 4.00 h)	(day 3, 4.30 h)	(day 3, 12.00 h)
	(day 2, 12.00 h)	day 3, 14.30 h	day 4, 4.00 h
	day 2, 20.00 h	(day 4, 4.30 h)	day 4, 20.00 h
	(day 3, 4.00 h)	day 4, 14.30 h	
	(day 3, 12.00 h)		
	day 3, 20.00 h		
Milking times in brackets: no treatment			

In the Omnygram[®] experiment with milking frequency of 2 times per day cows were treated at three successive milking intervals like recommended by the manufacturer (according to table 3). In the other two treatment trials cows were treated 3 times within 48 hours (table 4).

3.2. Analytical methods

3.2.1 Udder health

SCC was determined according to IDF Standard 148A:1995, Method C, Fluoro-opto-electronic method.

The bacteriological investigation was performed according to guidelines of the German Veterinary Association (DVG, 2000).

3.2.2 Qualitative detection of antibiotic residues

Microbiological inhibitor tests

The following commercially available microbial inhibitor tests with *B. stearothersophilus*¹ as test microorganisms were used:

Test with pH indicator

- Delvotest SP²

Tests with brilliant black as redox indicator:

- BR-AS³
- BR-AS special³
- BR-AS Brilliant³
- BRT³.

Test performance was according to the instructions of the manufacturer. The positive control sample contained 4 µg penicillin/kg. For confirmation of the presence of β-lactam antibiotics repeated analysis after addition of penicillinase (final concentration 50 U penicillinase⁴/ml milk) was applied.

The visual evaluation of test results was in four steps:

- 1 negative
- 2 colour slightly deviating from that of negative control sample
- 3 colour deviating from that of the negative control sample
- 4 colour at least as intensive as that of the positive control sample.

If applied as microbial inhibitor tests for quality payment purposes of ex-farm milk only step 4 is considered as positive. Steps 2,3 and 4 are considered as suspicious if test systems are applied as screening tests with subsequent analysis of antibiotics.

Microbial inhibitor tests were applied to screen milk samples used for the preparation of control samples applied in different analyses and for presence/absence test of inhibitors in the

¹ Current name: *Geobacillus stearothersophilus* due to recent taxonomic studies.

² DSM Food specialities, Giselherstrasse 12, D-44319 Dortmund/DE

³ Chr. Hansen GmbH, Grosse Drakenburger Strasse 93-97, D-31582 Nienburg/DE

⁴ E.C. 3.5.2.6, *B. cereus*, Sigma P 0389

samples of the excretion trials. The control samples were prepared according to ISO13969/IDF 183:2003.

Preliminary confirmation test - Beta Star³

The commercially available Beta Star test is a receptor assay for rapid detection of β -lactam antibiotic residues in milk.

The test is interpreted by visual comparison of a test band with a reference band. Results are coded in 4 levels; steps 2, 3 and 4 are interpreted as positive. The control samples were prepared according to ISO18330/IDF 188:2003.

Control samples

Negative and positive control samples spiked with the adequate substance and concentration of antibiotics were prepared in one batch and used for all examinations described. Preservation of the control samples was by lyophilization and storage at 6°C. The results of the microbial inhibitor tests and of the beta Star test were evaluated visually (steps 1, 2, 3, and 4).

Determination of detection limits

The description of IDF Standard 183/ISO 13969 was followed as close as adequate.

Tests applied: Delvo SP, BR-AS special, BRT - three different batches, one plate each;
Beta Star – one batch

Test samples: Inhibitor free raw milk from the Experimental Station of the Federal Dairy Research Centre. At least five different concentrations ($\mu\text{g}/\text{kg}$) of each compound were prepared and each concentration was dispensed in 14 tubes (Beta Star 5 tubes) and coded at random. The samples were used on the day of preparation.

Evaluation: Visual evaluation of test results by three persons \rightarrow 126 results per concentration (15 for Beta Star);
Photometric measurement of Delvo SP, BR-AS Special and BRT (\rightarrow 42 results per concentration) (Suhren and Luitz, 1995)

Detection limit: At least 95% positive results.

Detection limits were determined for the use of microbial inhibitor tests as screening tests as well as inhibitor tests. The following concentrations were used in spiked milk samples (Table 5).

Antibiotic	Microbial inhibitor tests					Receptor test
	BR AS	BR AS brilliant	BR AS special	BRT	Delvo SP	Beta Star
Cequinome	n.a.	n.a.	0, 50, 100, 150, 200, 250			0, 5, 10, 15, 20, 25
Penicillin	0, 1, 2, 3, 4, 5, 6, 7, 8					0, 1, 2, 3, 4, 5
Nafcillin	n.a.	0, 15, 30, 60, 90, 120			0, 7.5, 15, 30, 60	0, 5, 7.5, 15, 30
Dihydro-streptomycin	n.a.	0, 1 000, 2 500, 5 000				n.a.
PEN/NAF combinations	n.a.	n.a.	n.a.	n.a.	n.a.	0.5 PEN plus 0, 5, 7.5, 15, 30 NAF
Ampicillin	n.a.	n.a.	0, 2, 4, 6, 8			0, 2, 4, 6, 8
Colistin	n.a.	n.a.	0, 100, 500, 1 000, 5 000, 10 000, 50 000, 100 000			n.a.
n.a. = not applied						

In addition, detection limits were determined with incurred samples based on the results of the HPLC-analysis for treatment trials with Cobactan® LC (CEF) and Procain-Penicillin G. The results are summarized in tables 6 to 9.

Test	Spiked samples		Incurred samples	
	Inhibitor test	Screening test	Inhibitor test	Screening test
Delvo SP	150	100	100	70
BR-AS special	>250	>250	2000	800
BRT	>250	150	200	100
Beta-Star	12.5		10	

Of the microbial inhibitor tests the most sensitive test for the detection of CEF is Delvotest SP. However, the detection limit is much higher than the MRL of 20 µg/kg. By the receptor test Beta-Star CEF can be detected at MRL-level.

Test	Spiked samples		Incurred samples	
	Inhibitor test	Screening test	Inhibitor test	Screening test
Delvo SP	2	1	2	1
BR-AS special	2	1	3	2
BRT	3	2	4	4
BR-AS	4	3	n.d.	n.d.
BR-AS Brilliant	4	3	4	4
Beta-Star	2		2	
n.d. = not determined				

By all *B. stearothermophilus* tests the MRL for benzylpenicillin of 4 µg/kg can be detected. Delvo SP and BR-AS special are the most sensitive tests. The Beta-Star test also detects PEN at MRL-level.

Test	PEN		NAF		DHS	
	Inhibitor	Screening	Inhibitor	Screening	Inhibitor	Screening
Delvo SP	2	1	15	7.5	5 000	2 500
BR-AS sp.	2	1	15	15	5 000	2 500
BRT	3	2	15	15	n.d.	n.d.
BR AS Brilliant	4	3	30	30	n.d.	n.d.
Beta Star	2		30			
n.d. = not determined						

By all *B. stearothermophilus* tests the MRLs for PEN and NAF (4 µg/kg resp. 30 µg/kg) can be detected, whereas DHS cannot be detected at MRL level (200 µg/kg). The detection limit of the Beta Star for NAF was the same in samples spiked additionally with 0.5 µg PEN/kg.

	AMP		Colistin	
	Inhibitor	Screening	Inhibitor	Screening
Delvo SP	6	4	50 000	50 000
BR-AS sp.	4	4	10 000	5 000
BRT	6	4	50 000	50 000
Beta Star	6	4		

By all *B. stearothermophilus* tests the MRL for AMP of 4 µg/kg milk can be detected at least when applied as screening test. The BR-AS special test is the most sensitive test for this compound. Colistin is nearly not detected by these tests.

3.2.3 Quantitative detection of antibiotic residues - HPLC methods

For the identification and quantification of cefquinome, penicillin and dihydrostreptomycin published HPLC-methods were applied (Suhren and Knappstein, 1998, 2003) For detection of nafcillin and ampicillin the available methods were modified, for colistin a new method was developed. The apparatus consisted of a vacuum manifold (Adsorbex, SPU, Merck), a vacuum concentrator (JOTA-RVC, Christ), a gradient pump (Merck-Hitachi L-7100), a column thermostat (Merck Hitachi L-7360), an autosampler (Merck Hitachi L-7250), a UV-VIS detector (Merck Hitachi D-7420), a fluorescence detector (F1080-Merck), a reaction pump (Model 655-13, Merck) and an interface (Merck-Hitachi D-7000).

The experimental parameters are summarized in table 10

Table 10: Experimental parameters of liquid chromatographic methods for the detection of the different antibiotics

Experimental parameter	Cefquinome	Penicillin	Nafcillin	Dihydrostreptomycin	Ampicillin	Colistin
Internal standard	Sulfamethizole	Oxacillin	Oxacillin			
Sample preparation	Acid preparation, centrifugation, filtration	McIlvaine buffer, heating, cooling, centrifugation, fat discarding	McIlvaine buffer, heating, cooling, centrifugation, fat discarding	Acid preparation, centrifugation, fat discarding	Acid preparation, centrifugation, fat discarding, acid precipitation, filtration	Acid preparation, centrifugation, fat discarding, acid precipitation, filtration
Solid phase extraction						
Column	Cation exchange	C18	C18	C18	Benzolsulfonic acid column	Benzolsulfonic acid column
Elution	Potassium acetate/ acetonitrile	Acetonitrile/ methanole	Acetonitrile/ methanole	Acetonitrile/hexan-sulfonic-acid	Potassium borate/acetonitrile/ triethylamin	Potassium borate/ acetonitrile/ triethylamin, potassium acetate/acetonitrile
Concentration	4:1	20:1	18:1	10:1		
Liquid liquid extraction		Dichlormethane, potassium dihydrogenphosphate buffer	Dichlormethane, potassium dihydrogenphosphate buffer			
Dramatization/ Post column reaction		Benzoic acid anhydride, borate buffer, triazole, mercury(II) chloride	Benzoic acid anhydride, borate buffer, triazole, mercury(II) chloride	NaOH	Ortho-phthalaldehyde	Ortho-phthalaldehyde
LC-analysis						
Column	Superspher RP 18e	LiChrospher RP 18e	LiChrospher RP 18e	Superspher 60 RP Select B	Phenomex Prodigy C18	Chromolith RP 18e
Elution	Acetonitrile/ phosphate buffer	Acetate buffer, methanol, acetonitrile	Acetate buffer, methanol, acetonitrile	Hexan-sulfonic acid, Naphthquinone sulfonic acid, tri-ethylamine, acetonitrile	Phosphate buffer/ acetonitrile/ methanole	Phosphate buffer/acetonitrile
Wavelength (nm)	270	323	323	263/435	346/422	340/440
Retention time (min)	15/5	15/26	28/24	9	7	10/11
Recovery (%)	92/92	60/60	70	84-92	114	106
Repeatability, r ($\mu\text{g}/\text{kg}$)	0.9	0.2			4.5	53.0
Limit of detection	<2	<1	<1	29	1	9
Limit of quantification ($\mu\text{g}/\text{kg}$)	<3	<1	<1	54	2	14

Evaluation is via linear regression between peak areas and concentrations of aqueous standard solutions considering the concentration factor. The evaluated concentration in $\mu\text{g}/\text{kg}$ is corrected by the recovery factor of the corresponding internal standard.

By analysis of negative raw milk samples the limit of detection ($\text{LOD} = \text{XQ} + 3s$; with XQ = arithmetic mean, s = standard deviation) and the limit of quantification ($\text{LOQ} = \text{XQ} + 6s$) are derived.

3.3 Determination of withdrawal time

In order to compare the results of the experiments with different milking intervals different approaches to determine the withdrawal period were followed:

3.3.1 Pragmatic approach

- Pragmatic - average

For each experiment the mean concentration ($\mu\text{g}/\text{kg}$) of antibiotic in milk was calculated for every sampling time from the concentration of individual cows as determined by HPLC-methods. The sampling time after the last application was derived, where the average content fell below the respective MRL and did not exceed the MRL again. The mean was calculated from this time and the latest sampling time with an average value exceeding the MRL and defined as withdrawal time.

- Pragmatic - individual

The procedure described above was additionally applied for the individual cows of each experiment and the average withdrawal time calculated as mean from those values.

3.3.2 Time-to Safe-Concentration (TTSC) method

According to the Guidance of the European Agency for the Evaluation of Medicinal Products for the determination of withdrawal periods for milk (EMA, 1998) the TTSC-method calculates a tolerance limit for the number of milkings per animal. This tolerance limit is the time necessary for the residue concentration in milk of most animals to reach safe concentrations (i.e. the MRL). The method assumes a normal distribution after transformation of measured values onto the logarithmus naturalis (ln) scale. In order to derive the TTSC-points for each individual animal monotonic regression pre-processing of the data set was applied. Average and standard deviation of TTSC-points of the individual cows within experiment were calculated. According to the EMA-procedure the 95/95 tolerance limit was calculated by multiplication of the standard deviation with the indicated factor derived from the number of cows tested (e.g. in the case of 5 cows the limit factor is 4.210) and by addition of this product to the mean TTSC-value.

3.3.3 Regression model

Assuming a normal distribution after ln-transformation of measured concentrations quadratic as well as exponential regressions were calculated from the single concentrations and the time after last application within each experiment. From these regressions the intersections with the MRL-concentration and the upper limit of the 95 % confidence interval for an individual predicted value was computed.

3.4 Analysis of variance

In order to determine which factors have a systematic influence on the withdrawal time, an analysis of variance was carried out. For this purpose the GLM (General Linear Model) procedure of the statistic package SAS, release 8.01, was used.

The first time when the content fell below the MRL was used as dependent variable (y). The following influence factors were included into the analysis: milking frequency (3, 2, 1.5), days after calving (<=100 d, >100 d) and number of lactation (1, >1). Milk yield and SCC as continuous variables were used as covariate.

The linear model had the following form:

$$Y_{ijkl} = \mu + mf_i + dac_j + ln_k + b_1(X_{ijkl}) + b_2(X_{ijkl}) + e_{ijkl}$$

With:

Y_{ijkl} = dependent variable (first time (h) when the antibiotic content fell below the MRL)

μ = overall mean

mf_i = effect of the i^{th} milking frequency

dac_j = effect of the j^{th} days after calving

ln_k = effect of the k^{th} lactation number

b_1 = slope for milk yield

b_2 = slope for SCC

e_{ijkl} = random residual error

In addition, body weight (kg) was included as influencing factor for the Nafpenzal[®]MC and Omnygram[®] experiments.

4. Results

4.1 Cobactan[®] LC (Cefquinome)

4.1.1 Status of animals

Table 11: Cobactan[®] LC experiments – Status of animals					
Cow	No. of lactation	Days after calving	Milk yield per day [kg]*	SCC in composite milk [1000/ml]*	Mastitis pathogens
Milking frequency 3 times per day					
1792	2	149	30.3	24	RH,LH: CNS
1835	1	114	27.3	50	n.d.
1839	1	137	37.8	44	RH:CNS
1852	1	113	35.4	48	RH:CNS
2158	6	268	25.2	136	n.d.
Milking frequency 2 times per day (mishandled injectors)					
1743	4	116	36.4	16	n.d.
1796	2	156	23.6	25	n.d.
1818	2	168	28.6	19	n.d.
1829	1	76	31.8	62	RH,LH: CNS
1833	1	186	24.6	42	RH,LH: CNS
2158	6	299	22.7	113	n.d.
Milking frequency 2 times per day (repetition of experiment with correctly handled injectors)					
1774	4	101	34.0	138	RH: CNS
1818	3	104	32.4	18	n.d.
1827	2	95	30.6	31	n.d.
1835	2	93	25.0	61	RF,LF,LH: CNS
1859	1	82	32.8	15	n.d.
Milking frequency 1.5 times per day					
1761	3	147	28.2	28	n.d.
1786	3	122	28.8	87	n.d.
1804	2	153	33.2	37	RF: CNS
1815	2	81	33.4	35	RH: CNS
*average during experimental period, n.d. = not detected, CNS = coagulase-negative staphylococci; quarters: RF = right front, LF = left front, RH = right hind, LH = left hind					

Cow No. 2158 was included in trials with milking 2 times per day due to prolonged excretion in experiment with milking frequency of 3 times per day.

The experiment with milking 2 times per day had to be repeated because udder injectors for the second of 3 treatments were accidentally stored above 30 °C.

One cow had to be excluded from the experiment with milking frequency 1.5 times per day after the anamnestic phase because of systemic illness.

4.1.2 SCC

In figure 1 SCC in cow composite milk samples is summarized as average per group.

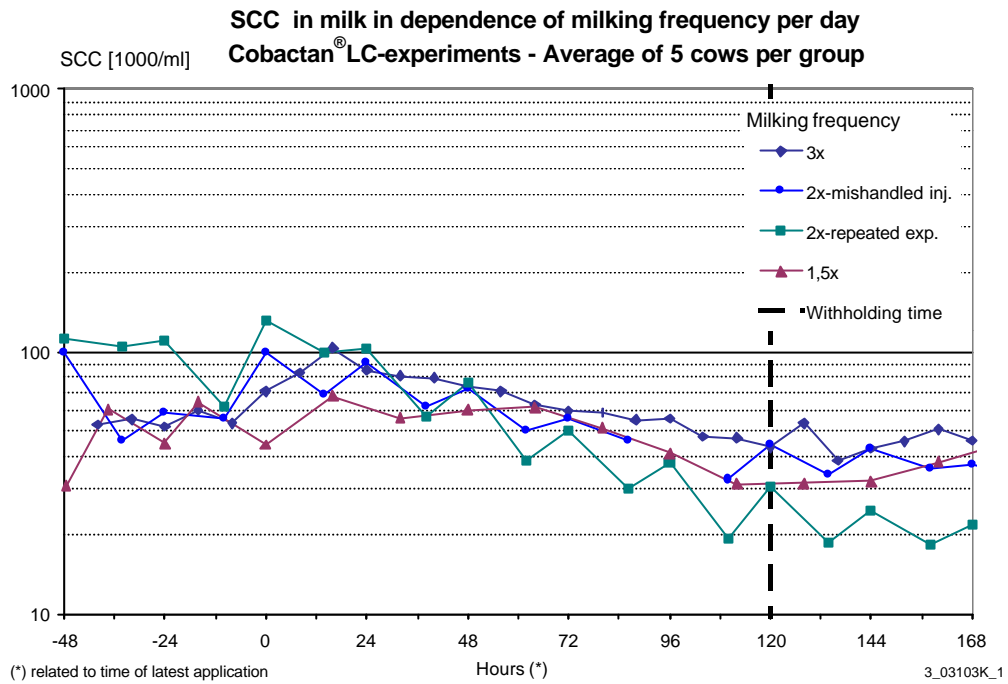


Figure 1

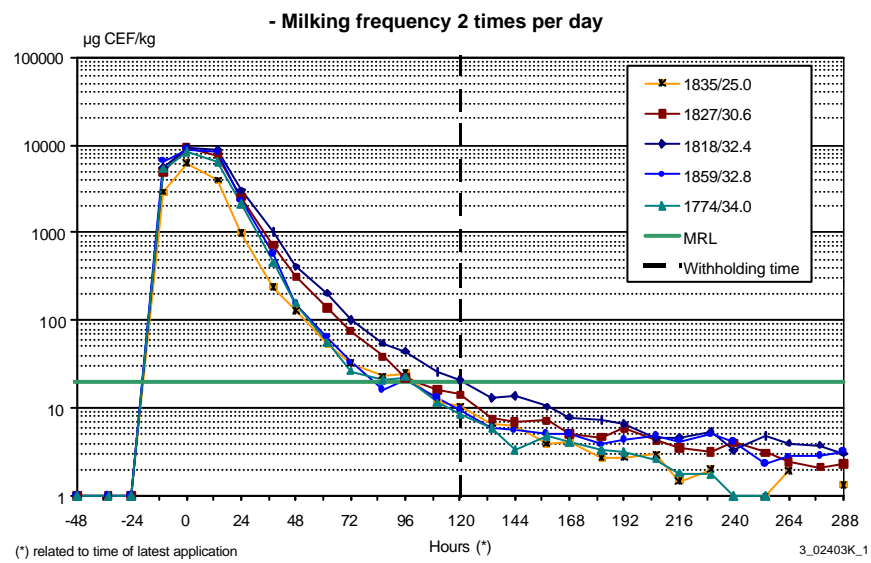
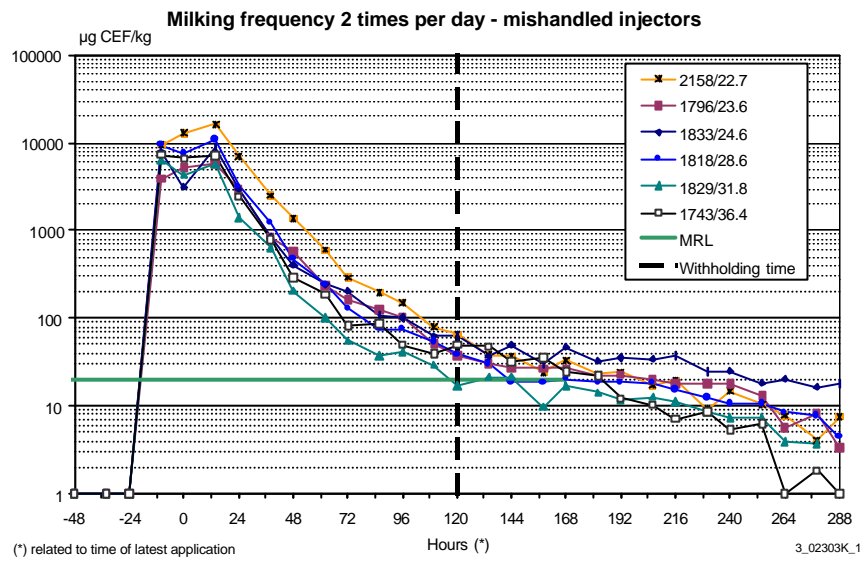
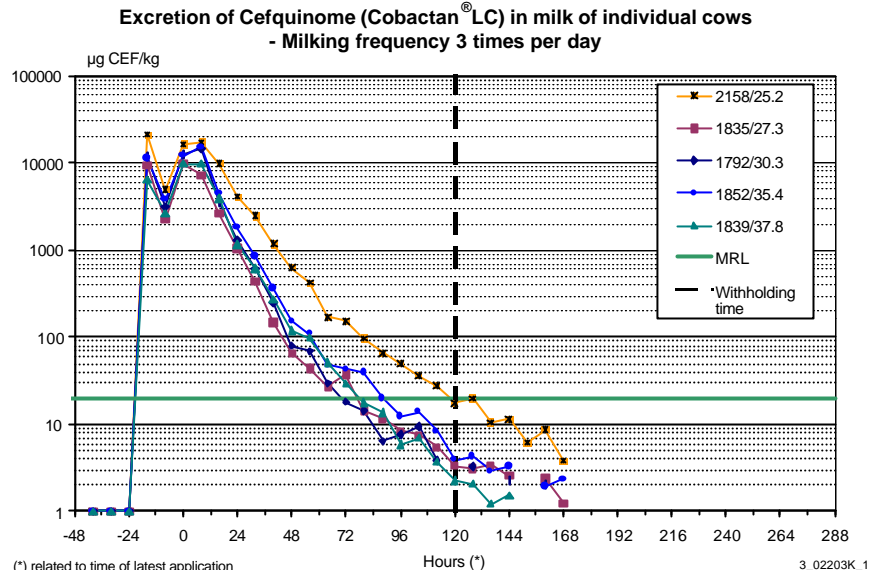
4.1.3 Excretion

In table 12 the excreted quantity of CEF via milk with respect to the milking interval is summarized per cow and group.

Table 12: CEF excretion via milk in dependence of milking interval			
Cow no.	Milk yield [kg]	Excreted CEF	
		in mg	in % of applied*
Milking frequency: 3 times per day			
1839	37.8	441	49.0
1852	35.4	592	65.8
1792	30.3	485	53.9
1835	27.3	305	33.9
2158	25.2	597	66.3
Average	31.2	484	53.7
Milking frequency: 2 times per day (mishandled injectors)			
1743	36.4	426	47.3
1829	31.8	337	37.5
1818	28.6	521	57.9
1833	24.6	341	37.9
1796	23.6	250	27.8
2158	22.7	597	66.3
Average	28.0	412	45.8
Milking frequency: 2 times per day			
1774	34.0	397	44.1
1859	32.8	465	51.7
1818	32.4	498	55.3
1827	30.6	406	45.1
1835	25.0	173	19.2
Average	31.0	388	43.1
Milking frequency: 1.5 times per day			
1815	33.4	468	52.0
1804	33.2	463	51.4
1786	28.8	367	40.8
1761	28.2	555	61.7
Average	30.9	463	51.5
*100 % = 900 mg			

4.1.4 Withdrawal time

The excretion curves of the individual cows within each experiment and the average curves are demonstrated in figures 2.1-2.4 resp. 3.



Figures 2.1-2.3

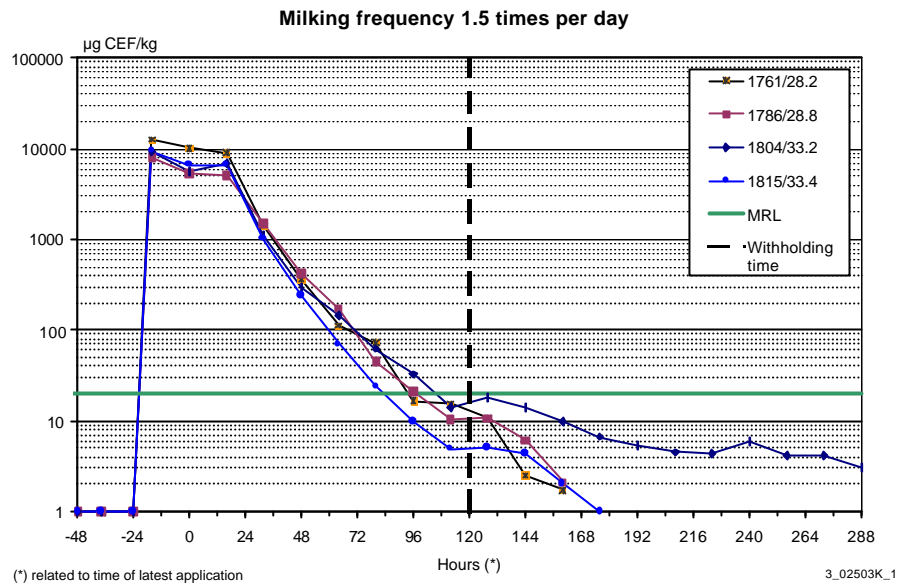


Figure 2.4

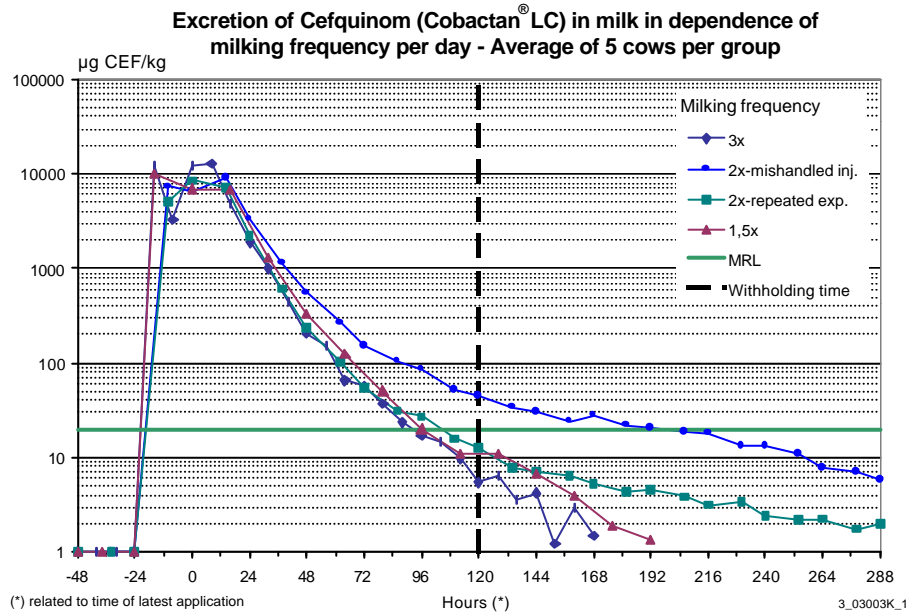


Figure 3

The quadratic and exponential regressions for each experiment are presented in figure 4.

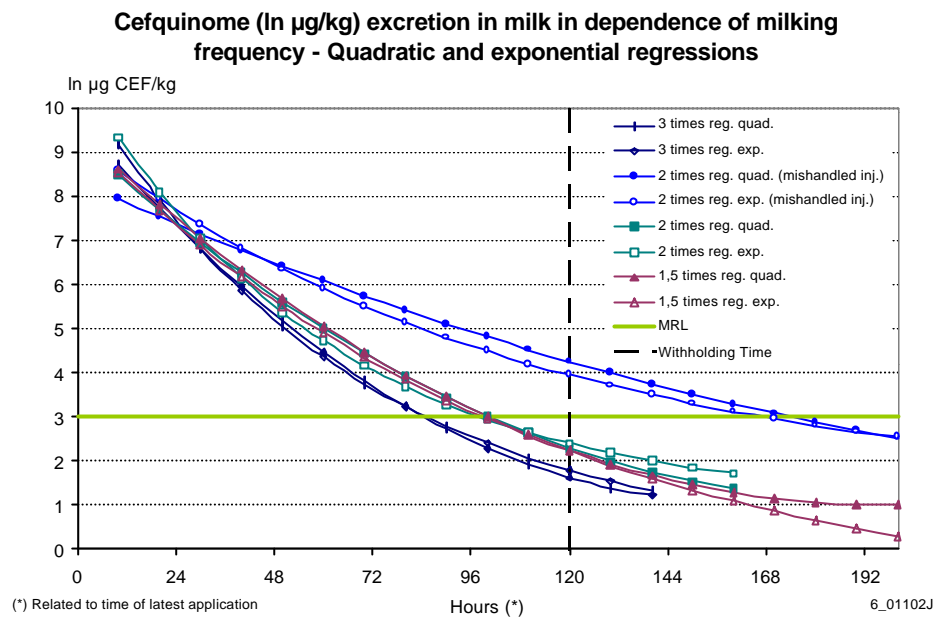


Figure 4

The results of the determination of withdrawal times by different approaches is summarized in table 13.

Table 13: Withdrawal time of CEF (in hours) in dependence of milking frequency – summarized results from pragmatic approach, TTSC and regression models		
Official withdrawal time: 120 h		
Approach	Mean	95/95 tolerance limit/ 95% confidence interval
Milking frequency: 3 times per day		
Pragmatic - individual	88	
Pragmatic -average	92	
TTSC ^a	87	197
Quadratic regression ^b	84	121
Exponent. regression ^b	84	127
Milking frequency: 2 times per day (mishandled injectors)		
Pragmatic - individual	199	
Pragmatic -average	199	
TTSC ^a	187	547
Quadratic regression ^b	174	298
Exponent. regression ^b	165	305
Milking frequency 2 times per day		
Pragmatic - individual	108	
Pragmatic -average	103	
TTSC ^a	109	211
Quadratic regression ^b	102	132
Exponent. regression ^b	97	129
Milking frequency: 1.5 times per day		
Pragmatic – individual	96	
Pragmatic –average	104	
TTSC ^a	104	164
Quadratic regression ^b	100	137
Exponent. regression ^b	103	142
^a 95/95 tolerance limit, ^b 95 % confidence interval		

4.1.5 Analysis of variance

The data from the experiment with mishandled injectors were excluded from the analysis of variance.

The model accounted for 63% of the deviance of excretion time, but none of the predictors was significant. The differences between milking frequencies were therefore not significant (table 14).

The experiment with 3 milkings per day had the lowest withdrawal time, but there was no constantly descending withdrawal time with increasing milking frequency. Milking twice a day showed a higher withdrawal time than with 1.5 milkings per day.

Tab.14: Withdrawal time (in hours) for CEF (Least Square Means (LSQ_M) and standard error)		
Milking frequency	LSQ_M± se	sign. *
3 times	80.3 ± 20.0	a
2 times	108.8 ± 11.1	a
1.5 times	95.9 ± 15.5	a
* Different letters show significant differences (p<0.05)		

The analysis was repeated without data of cow no. 2158, which showed a markedly deviating excretion time from the other cows in the group milked 3 times per day (see figure 2.1). Using only the milking frequency and the days after calving, the generalized linear model accounted for 73% of the variance.

There were significant differences (p<0.01) between the milking frequency 3 times per day and the other tested frequencies, but the difference between 2 and 1.5 milking per day was not significant (table 15).

Tab.15: Withdrawal time (in hours) of CEF (Least Square Means (LSQ_M) and standard error) – Calculations without data of cow 2158		
Milking frequency	LSQ_M± se	sign.*
3 times	71.8 ± 6.5	a
2 times	111.6 ± 4.8	b
1.5 times	99.9 ± 5.6	b
* Different letters show significant differences (p<0.05)		

The difference between the classes for the days after calving was slightly significant (p<0.1%). The group in early lactation (<100 days after calving) reached the concentration below the MRL quicker than the cows in the other class (LSQ_M ± se : 86.2 ± 6.2 to 102.7 ± 3.7).

Due to the analysis of variance SCC had no significant influence on the excretion time. Therefore no further analysis was performed on the mastitis pathogens involved.

4.2 Procain-Penicillin G

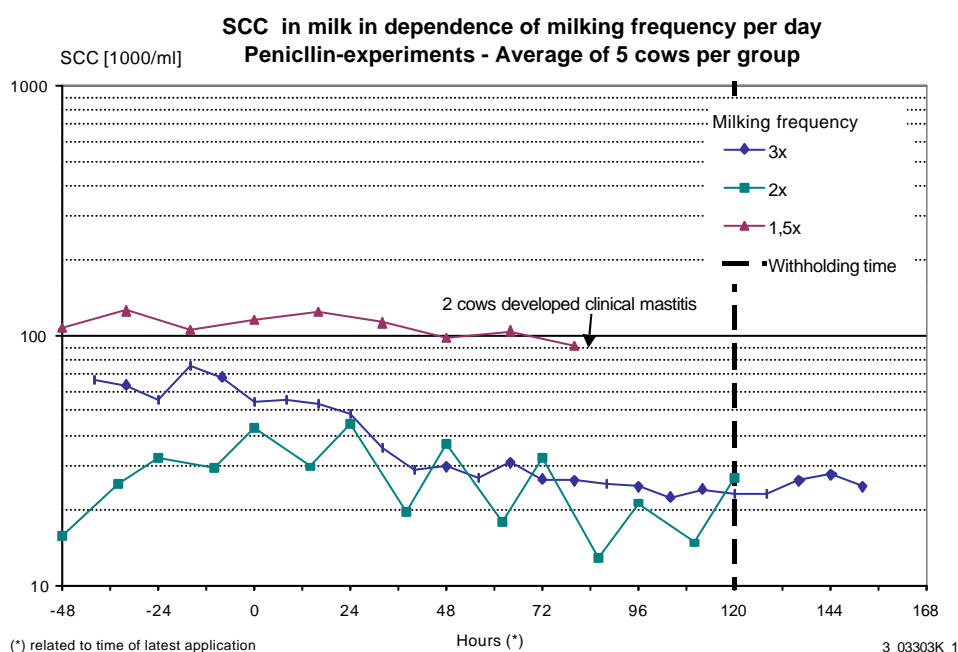
4.2.1 Status of animals

Table 16: Procain-Penicillin G-experiments – Status of animals					
Cow	No. of lactation	Days after calving	Milk yield per day [kg]*	SCC in composite milk [1000/ml]*	Mastitis pathogens
Milking frequency 3 times per day					
1731	4	162	33.9	43	n.d.
1810	2	176	23.5	58	LH: CNS
1827	1	133	29.1	36	n.d.
1831	1	138	38.6	26	n.d.
1834	1	96	32.9	53	RH: CNS
Milking frequency 2 times per day)					
1796	3	41	31.4	21	n.d.
1833	2	110	33.6	26	n.d.
1869	1	46	29.0	34	n.d.
1870	1	56	32.7	25	n.d.
1880	1	54	24.4	26	n.d.
Milking frequency 1.5 times per day					
1697	5	111	32.4	17	n.d.
1716	5	82	33.1	391	n.d.
1763	3	94	32.5	34	n.d.
1824	1	190	26.9	25	n.d.
1848	1	215	22.6	71	n.d.

*average during experimental period, n.d. = not detected, CNS = coagulase-negative staphylococci; quarters: RF = right front, LF = left front, RH = right hind, LH = left hind

4.2.2 SCC

Figure 5:



The average SCC in cow composite milk is shown in figure 5.

4.2.3 Excretion

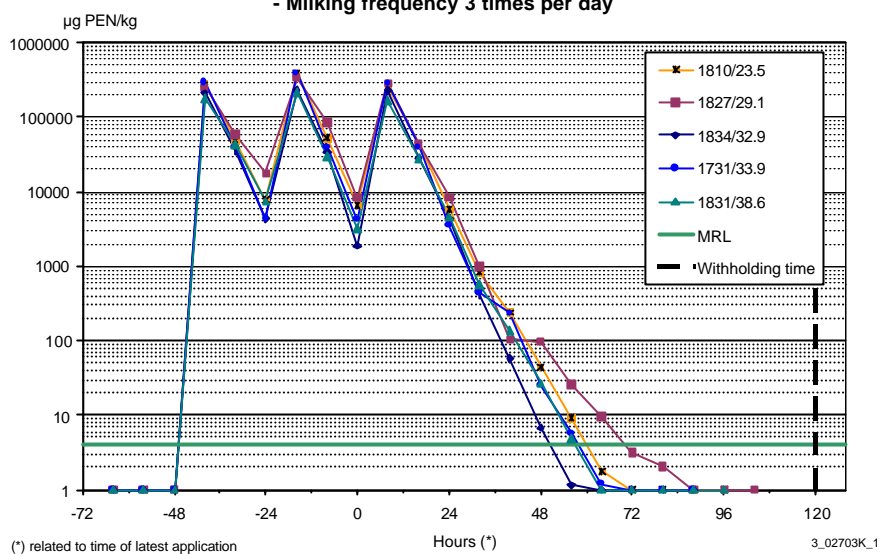
In table 17 the excreted quantity of PEN via milk with respect to the milking interval is summarized per cow and group:

Tab. 17: PEN excretion via milk in dependence of milking interval			
Cow no.	Milk yield [kg]	Excreted PEN	
		mg	in % of applied*
Milking frequency: 3 times per day			
1831	38.6	9 244	40.6
1731	33.9	13 060	57.3
1834	32.9	8 760	38.5
1827	29.1	11 055	48.5
1810	23.5	8 293	36.4
Average	31.6	10 078	44.2
Milking frequency: 2 times per day			
1833	33.6	7 439	32.7
1870	32.7	8 206	36.0
1796	31.4	5 474	24.0
1869	29.0	6 548	28.7
1880	24.4	10 190	44.7
Average	30.2	7 572	33.2
Milking frequency: 1.5 times per day			
1763	32.5	9 695	42.6
1716	33.1	9 793	43.0
1697	32.4	7 654	33.6
1824	26.9	7 856	34.5
1848	22.6	7 042	30.9
Average	29.5*	8 408	36.9
* 100 % = 22 776 mg			

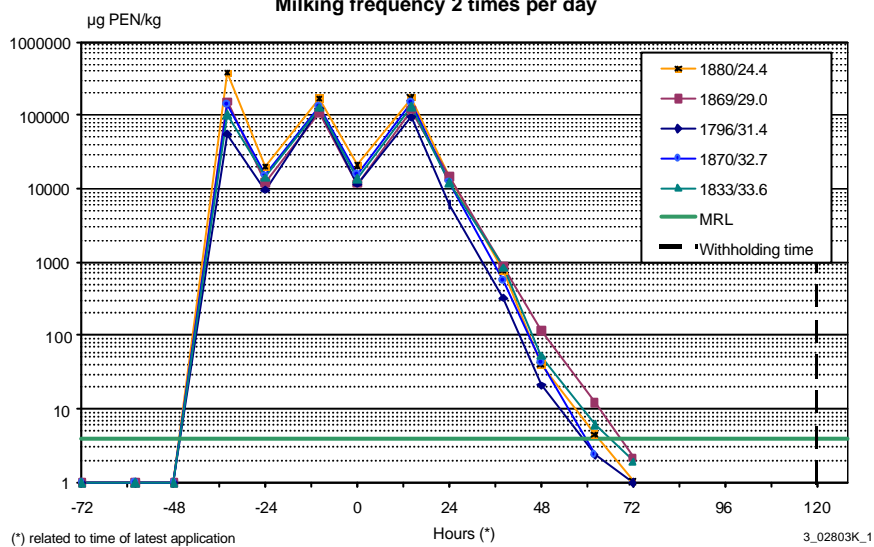
4.2.4 Withdrawal time

In figures 6.1-6.3 the excretion curves of the individual cows within each experiment and in figure 7 the average curves are demonstrated.

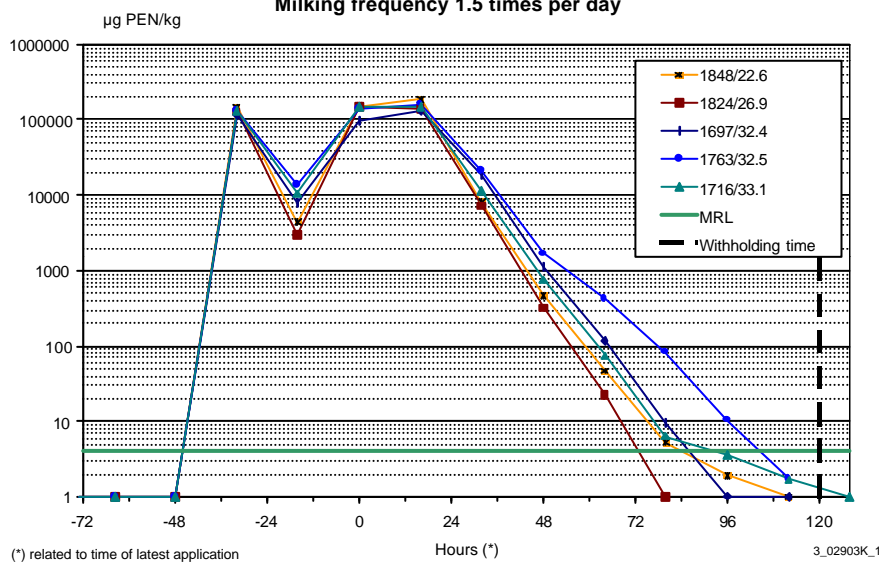
**Excretion of Penicillin in milk of individual cows
- Milking frequency 3 times per day**



Milking frequency 2 times per day



Milking frequency 1.5 times per day



Figures 6.1-6.3

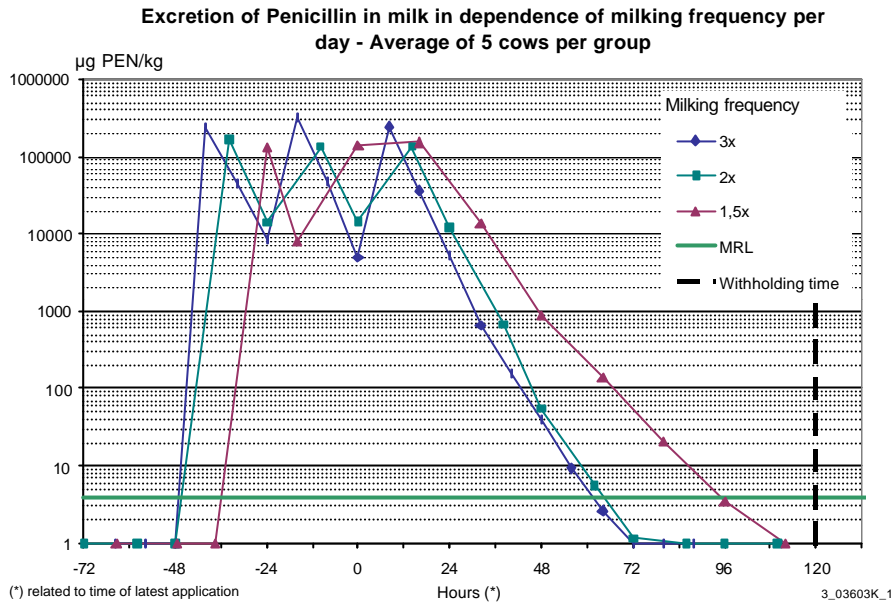


Figure 7

The quadratic and exponential regressions for each experiment are summarized in figure 8.

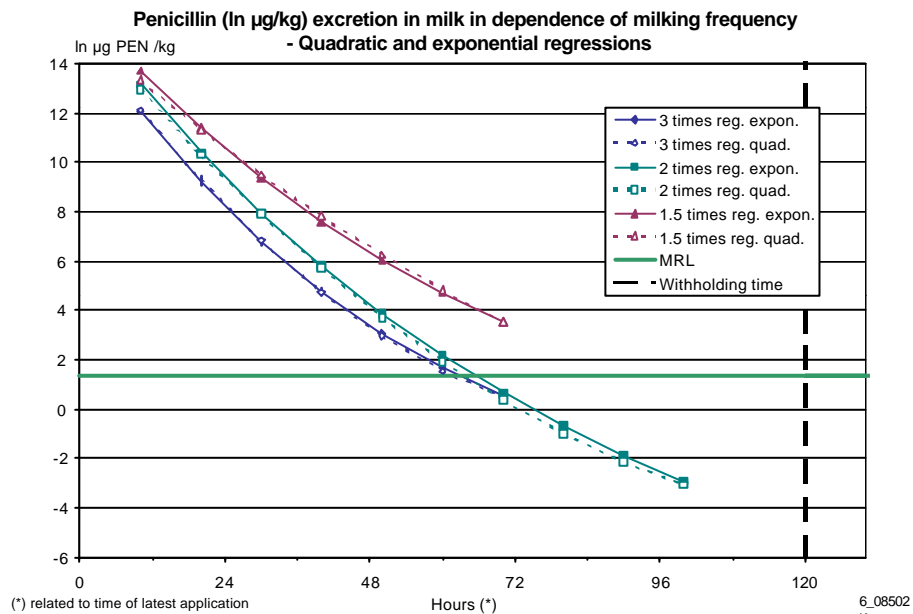


Figure 8

The results of the determination of withdrawal times by the different approaches are compared in table 18.

Table 18: Withdrawal time of PEN (in hours) in dependence of milking frequency – summarized results from pragmatic approach, TTSC and regression models		
Official withdrawal time: 120 h		
Approach	Mean	95/95 tolerance limit/ 95% confidence interval
Milking frequency: 3 times per day		
Pragmatic - individual	60	
Pragmatic -average	60	
TTSC ^a	64	93
Quadratic regression ^b	61	82
Exponent. regression ^b	62	78
Milking frequency 2 times per day		
Pragmatic - individual	62	
Pragmatic -average	67	
TTSC ^a	68	96
Quadratic regression ^b	63	72
Exponent. regression ^b	64	73
Milking frequency: 1.5 times per day		
Pragmatic - individual	91	
Pragmatic -average	88	
TTSC ^a	95	158
Quadratic regression ^b	90	113
Exponent. regression ^b	92	120
^a 95/95 tolerance limit, ^b 95 % confidence interval		

4.2.5 Analysis of variance

The model accounted for 84 % of the deviance of excretion time, but only the milking frequency had a significant influence on the withdrawal time. Using only the milking frequency 81% of the variance can be explained.

The withdrawal time decreased with increasing milking frequency. But only the differences between milking 3 times per day and milking 1.5 times per day were significant (table 19).

Table 19: Withdrawal time (in hours) for PEN (Least Square Means (LSQ_M) and standard error)		
Milking frequency	LSQ_M ± se	sign.*
3 times	64.7 ± 4.6	a
2 times	65.7 ± 4.5	a b
1.5 times	96.3 ± 4.5	b
* Different letters show significant differences (p<0.05)		

4.3 Nafpenzal[®] MC (Penicillin, Nafcillin, Dihydrostreptomycin)

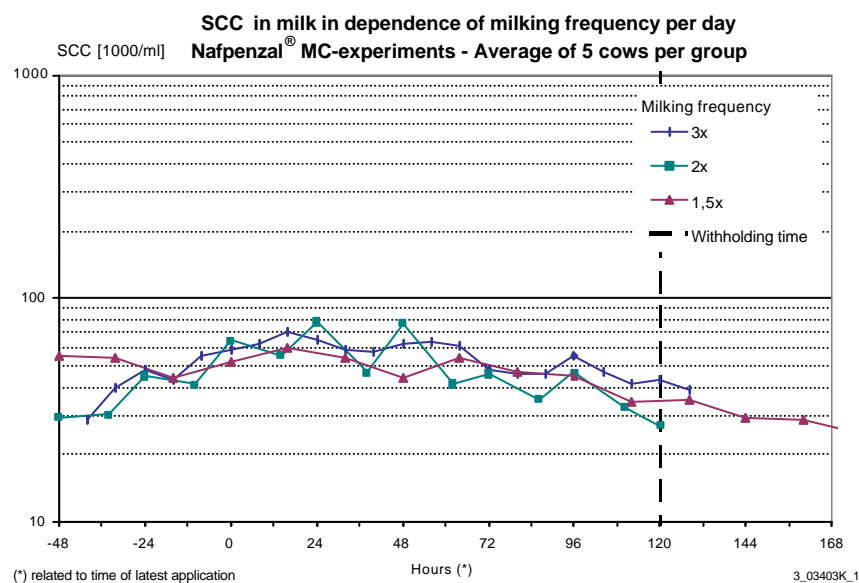
4.3.1 Status of animals

Tab. 20: Nafpenzal [®] MC-experiments – Status of animals						
Cow.	No. of lactation	Days after calving	Body weight [kg]	Milk yield per day [kg]	SCC in composite milk [1000/ml]*	Mastitis pathogens
Milking frequency 3 times per day						
1751	5	84	684	31.5	56	LH: CNS
1831	2	140	767	27.3	51	LF: CNS
1842	2	101	732	35.1	17	n.d
1859	1	185	690	29.4	73	n.d
1880	1	173	534	25.2	56	RH: CNS
Milking frequency 2 times per day						
1818	3	185	733	26.4	53	n.d.
1827	2	176	660	23.8	45	n.d
1829	2	81	645	32.4	41	LF+RH: CNS
1846	2	110	652	30.6	51	n.d
1867	1	143	585	21.4	36	n.d
Milking frequency 1.5 times per day						
1786	4	104	698	30.9	36	n.d
1830	2	165	745	30.7	41	n.d
1855	1	186	676	35.6	46	LH: CNS
1863	1	231	724	26.9	66	LF: CNS
1870	1	215	595	26.6	42	LH: Coryneform

*average during experimental period, n.d. = not detected, CNS = coagulase-negative staphylococci; quarters: RF = right front, LF = left front, RH = right hind, LH = left hind

4.3.2 SCC

Figure 9



In figure 9 SCC in cow composite milk samples is summarized as average per group.

4.3.3 Excretion

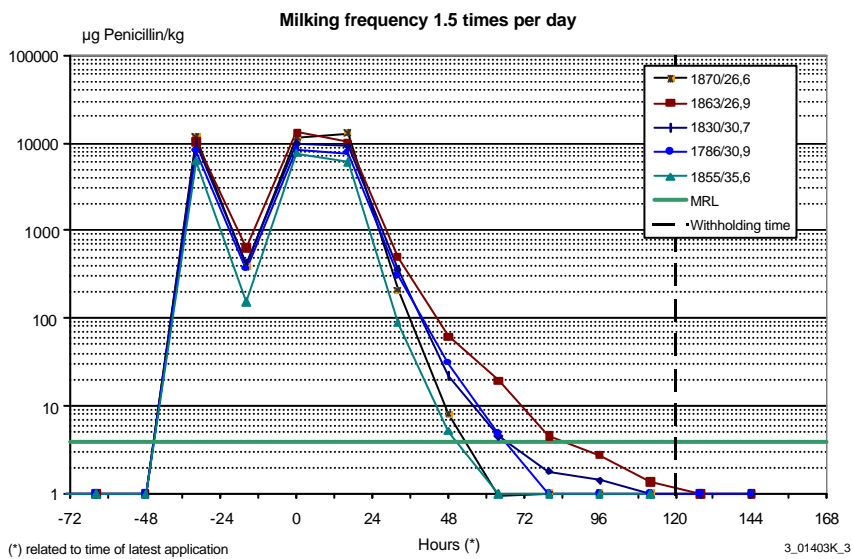
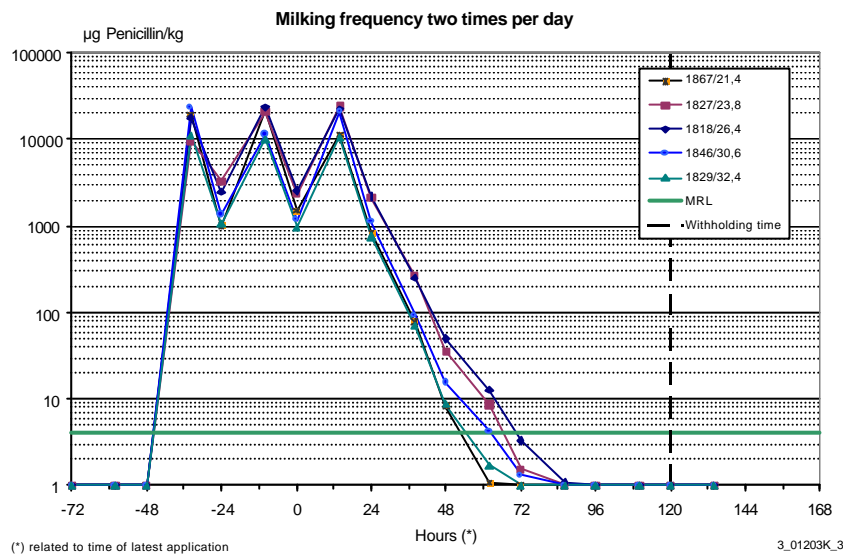
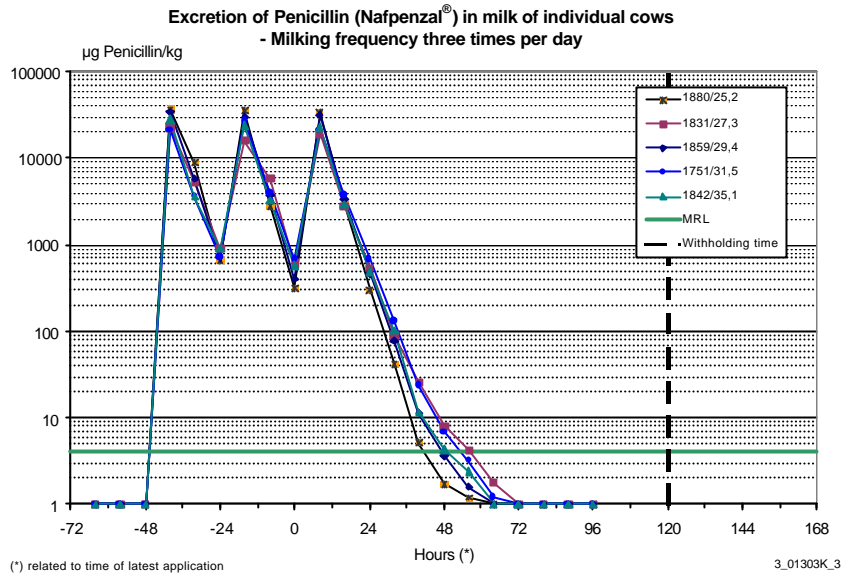
In table 21 the excreted quantities of PEN, NAF and DHS via milk with respect to the milking interval are summarized per cow and group:

Table 21: Nafpenzal[®] MC experiments: PEN, NAF and DHS excretion via milk in dependence of milking interval							
Cow	Milk yield [kg]	Excreted PEN		Excreted NAF		Excreted DHS	
		mg	% of applied*	mg	% of applied*	mg	% of applied*
Milking frequency: 3 times per day							
1842	35.1	1 018	47.1	448	37.3	1097	91.4
1751	31.5	885	41.0	302	25.2	958	79.8
1859	29.4	1 068	49.4	494	41.2	1 065	88.8
1831	27.3	682	31.6	261	21.8	718	59.8
1880	25.2	988	45.7	448	37.3	957	79.8
Average	29.7	928	43.0	391	32.6	959	79.9
Milking frequency: 2 times per day							
1829	32.4	642	29.7	237	19.8	797	66.4
1846	30.6	960	44.4	394	32.8	975	81.3
1818	26.4	1 078	49.9	348	29.0	1 219	101.5
1827	23.8	844	39.1	313	26.1	979	81.5
1867	21.4	692	32.0	283	23.6	585	48.8
Average	26.8	843	39.0	315	26.3	911	75.9
Milking frequency: 1.5 times per day							
1855	35.6	371	17.2	162	13.5	429	37.8
1786	30.9	514	23.8	171	14.3	647	53.9
1830	30.7	613	28.4	227	18.9	658	54.8
1863	26.9	637	29.5	248	20.7	662	55.1
1870	26.6	643	29.8	265	22.0	693	57.8
Average	30.1	556	25.7	215	17.9	618	51.5
* 100 % = 2160 mg PEN, 1200 mg NAF, 1200 mg DHS							

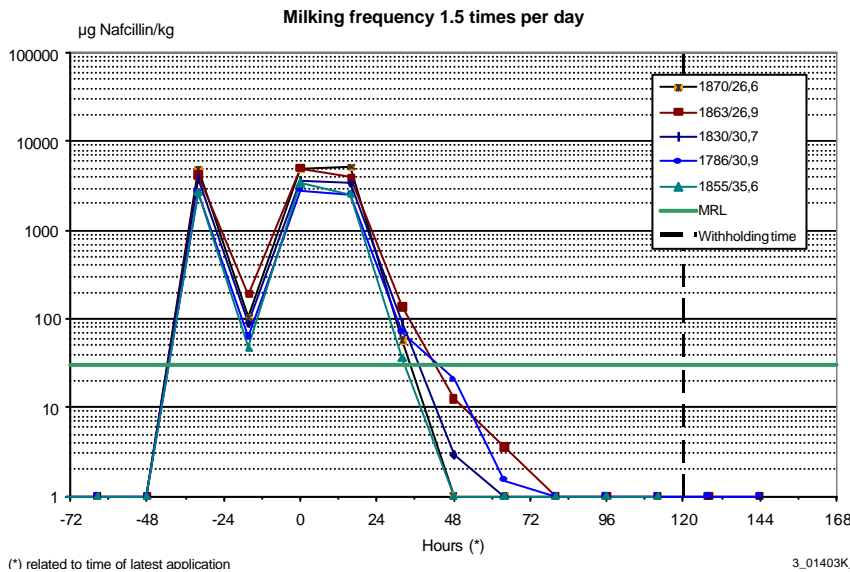
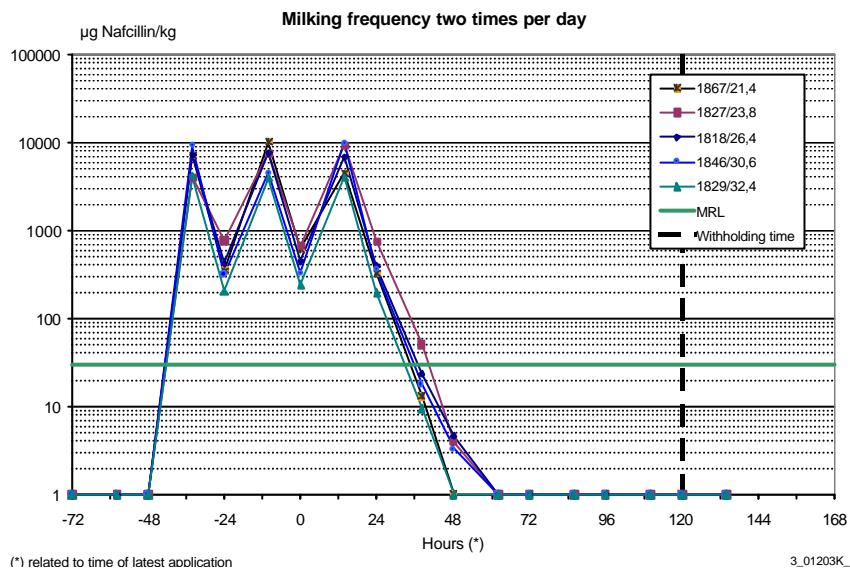
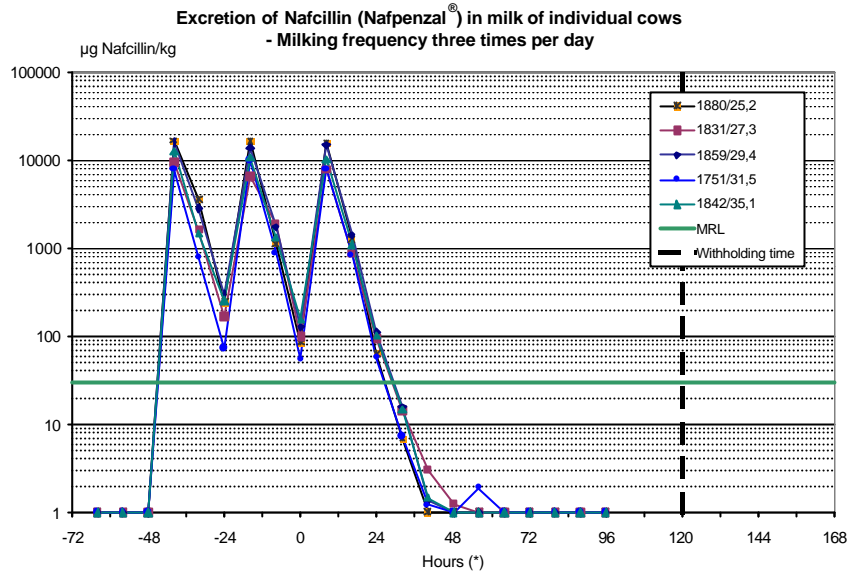
4.3.4 *Withdrawal time*

In fig 10.1-12.3 the excretion curves of the individual cows within each experiment is demonstrated separately for each antibiotic compound. The average curves are demonstrated in figures 13.1-13.3. The withdrawal times differ with respect to the country where the drug is registered (see table 2). In the graphs the withdrawal time valid in NL is indicated.

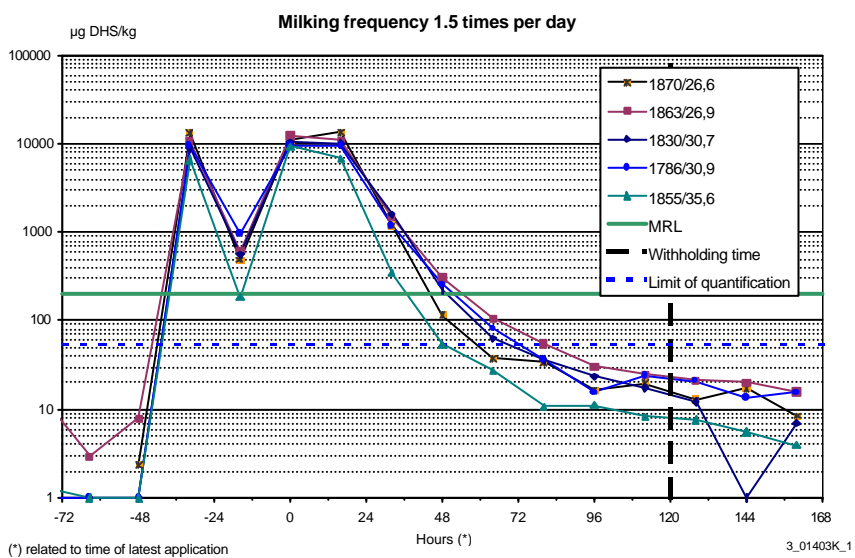
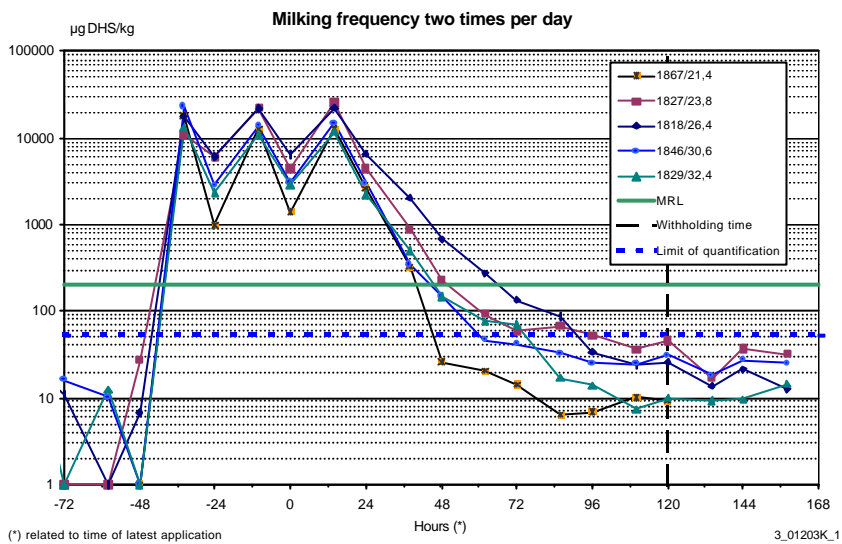
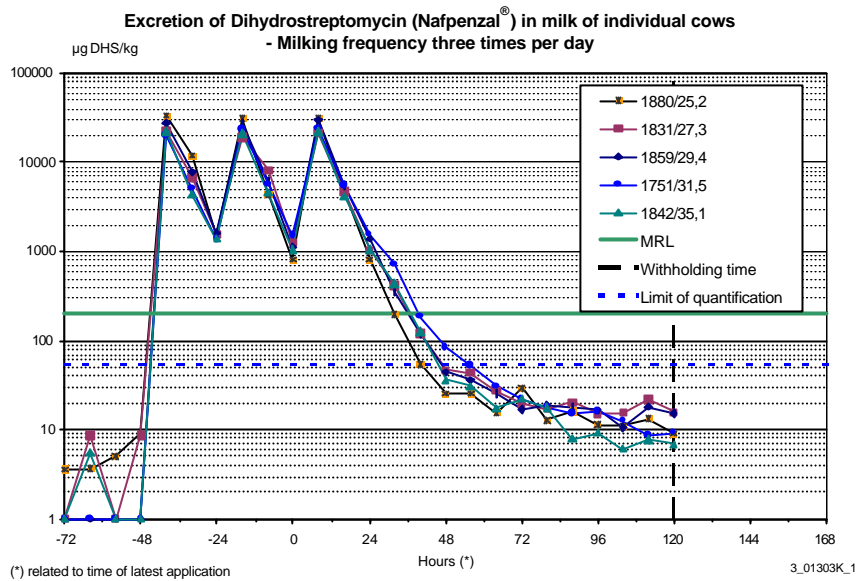
The quadratic and exponential regressions for each substance are presented in figures 14.1-14.3.



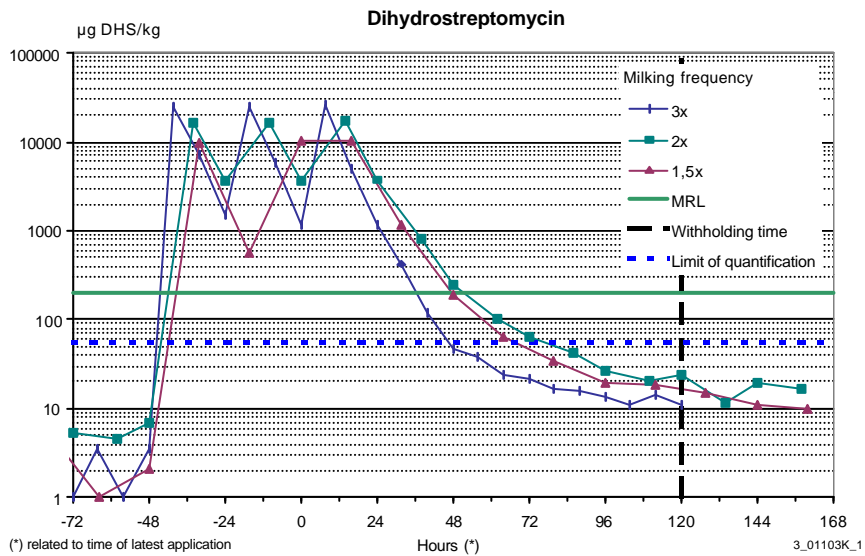
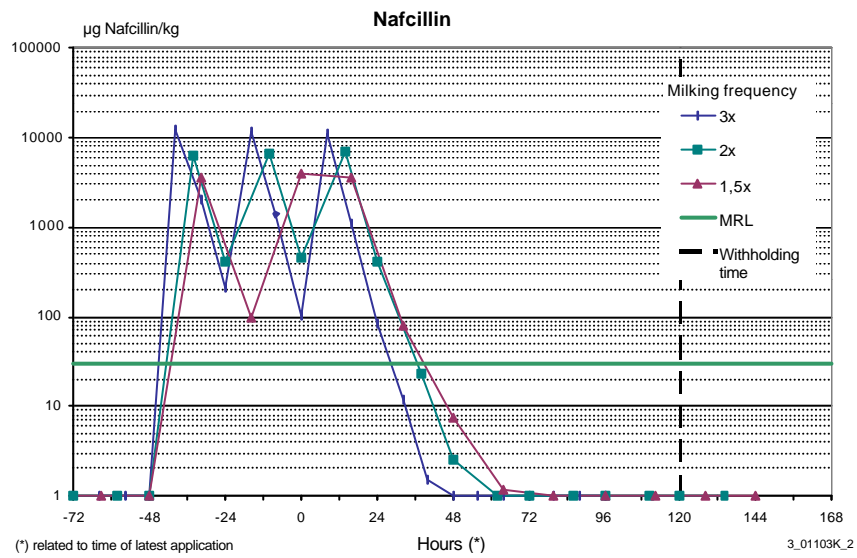
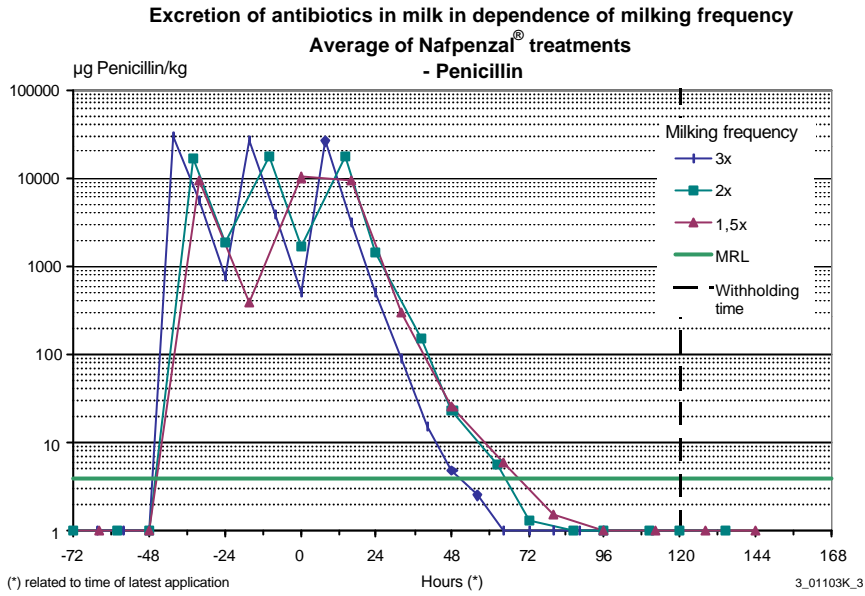
Figures 10.1-10.3



Figures 11.1-11.3

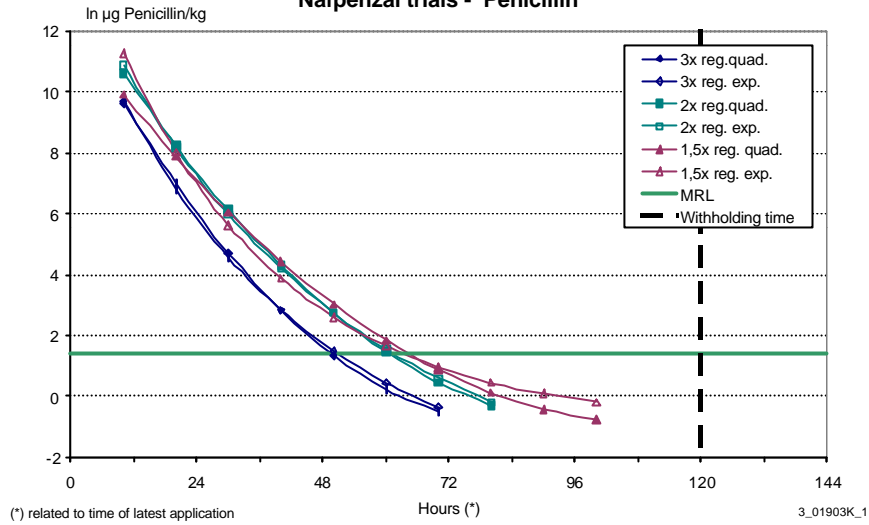


Figures 12.1-12.3

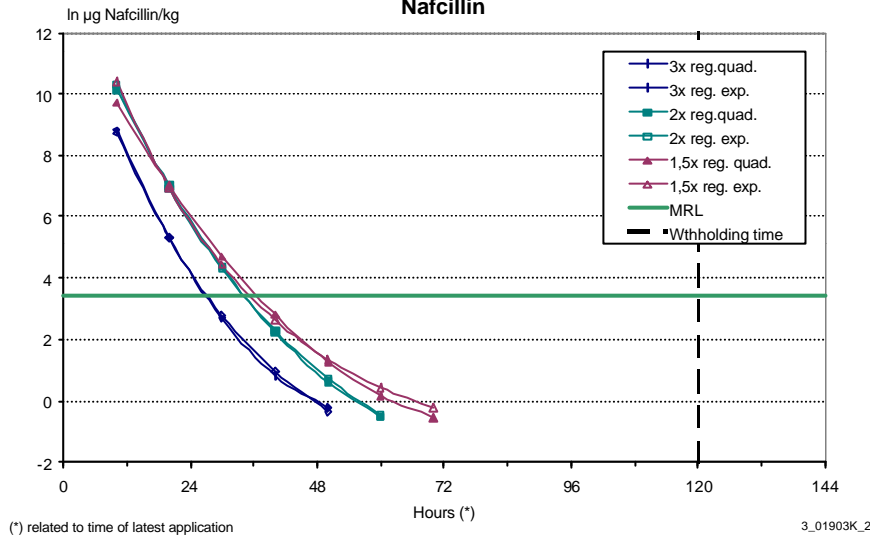


Figures 13.1-13.3

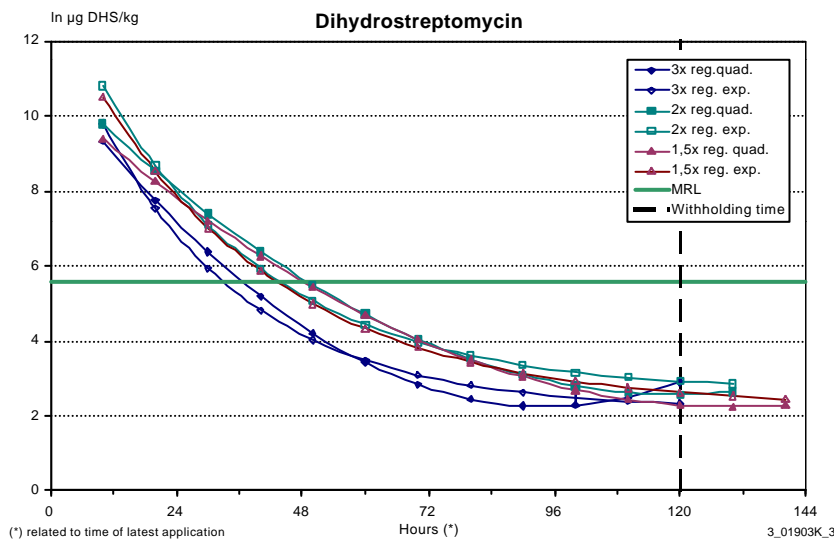
Excretion of antibiotics in milk in dependence of milking frequency - Quadratic and exponential regressions
Nafpenzal trials - Penicillin



Nafcillin



Dihydrostreptomycin



Figures 14.1-14.3

Table 22 summarizes the results of the determination of withdrawal times by different approaches.

Table 22: Withdrawal time (in hours) of Nafpenzal[®] MC in dependence of milking frequency – summarized results from pragmatic approach, TTSC and regression model (Official withdrawal time: UK 72 h, NL 120 h)		
PEN	Mean	95/95 tolerance limit/ 95% confidence interval
Milking frequency: 3 times per day		
Pragmatic - individual	50	
Pragmatic -average	52	
TTSC ^a	54	90
Quadratic regression ^b	50	57
Exponent. regression ^b	51	61
Milking frequency 2 times per day		
Pragmatic – individual	62	
Pragmatic –average	67	
TTSC ^a	68	96
Quadratic regression ^b	61	75
Exponent. regression ^b	62	80
Milking frequency: 1.5 times per day		
Pragmatic – individual	69	
Pragmatic –average	72	
TTSC ^a	76	157
Quadratic regression ^b	65	100
Exponent. regression ^b	64	120
NAF	Mean	95/95 tolerance limit/ 95% confidence interval
Milking frequency: 3 times per day		
Pragmatic - individual	28	
Pragmatic -average	28	
TTSC ^a	32	32
Quadratic regression ^b	27	31
Exponent. regression ^b	27	34
Milking frequency 2 times per day		
Pragmatic – individual	33	
Pragmatic –average	31	
TTSC ^a	40	62
Quadratic regression ^b	34	39
Exponent. regression ^b	34	41
Milking frequency: 1.5 times per day		
Pragmatic - individual	40	
Pragmatic -average	40	
TTSC ^a	48	48
Quadratic regression ^b	37	46
Exponent. regression ^b	35	48

Table 22 - continued		
DHS	Mean	95/95 tolerance limit/ 95% confidence interval
Milking frequency: 3 times per day		
Pragmatic - individual	36	
Pragmatic -average	36	
TTSC ^a	38	58
Quadratic regression ^b	39	48
Exponent. regression ^b	35	42
Milking frequency 2 times per day		
Pragmatic - individual	50	
Pragmatic -average	55	
TTSC ^a	58	122
Quadratic regression ^b	52	75
Exponent. regression ^b	47	73
Milking frequency: 1.5 times per day		
Pragmatic - individual	50	
Pragmatic -average	40	
TTSC ^a	57	111
Quadratic regression ^b	52	70
Exponent. regression ^b	46	61
^a 95/95 tolerance limit, ^b 95 % confidence interval		

The longest withdrawal periods are necessary for penicillin whereas the shortest excretion time period was observed for nafcillin.

4.3.5 Analysis of variance

Without variance factor body weight

Penicillin

The model accounted for 77 % of deviance in excretion time. PEN showed similar results like DHS. Milking frequency ($p < 0.01$) and lactation number ($p < 0.05$) had significant influence on the withdrawal time. For milking frequency only the differences between the milking frequency 3 times per day and 1.5 times per day were significant. The predicted values are listed in table 24.

Table 24: Withdrawal time (in hours) for PEN (Least Square Means (LSQ_M) and standard error)		
Milking frequency	LSQ_M ± se	sign.*
3 times	51.1 ± 4.5	a
2 times	61.4 ± 4.7	a b
1.5 times	76.7 ± 5.3	b
* Different letters show significant differences ($p < 0.05$)		

The predicted withholding period for cows in first lactation (56.1 hours) was shorter than for cows with higher lactation number >1 (70.1 hours).

Nafcillin

For NAF only the milking frequency ($p < 0.01$) showed a significant influence on the withdrawal time. Due to the low variation between the withdrawal times 91 % of the deviance of time could be explained by the model. Withdrawal time decreased with increasing milking frequency. Differences between all three milking frequencies were significant. The predicted values are listed in table 25.

Table 25: Withdrawal time (in hours) for NAF (Least Square Means (LSQ_M) and standard error)		
Milking frequency	LSQ_M ± se	sign.*
3 times	31.8 ± 1.6	a
2 times	38.8 ± 1.7	b
1.5 times	48.0 ± 1.9	c
* Different letters show significant differences ($p < 0.01$)		

Dihydrostreptomycin

The milking frequency ($p < 0.01$) and the lactation number ($p < 0.05$) had a significant influence on the withdrawal time. The model accounted for 80 % of deviance in time. The withdrawal time decreased with increasing milking frequency. Only the differences between the milking frequency of 3 times per day and the other milking frequencies were significant. The predicted values are listed in table 26.

Table 26: Withdrawal time (in hours) for DHS (Least Square Means (LSQ_M) and standard error)		
Milking frequency	LSQ_M ± se	sign.*
3 times	34.8 ± 3.9	a
2 times	48.9 ± 4.0	b
1.5 times	56.7 ± 4.6	b
* Different letters show significant differences ($p < 0.05$)		

Including the factor body weight

In order to determine whether the body weight has an influence on the withdrawal time, the model was extended by this continuous variable.

Including body weight slightly increased the calculated withdrawal times for all antibiotics (as LSQ_M), but had no influence on significance of differences between milking frequencies.

Penicillin

For PEN lactation number had no significant influence on the withdrawal time when body weight was included. Only milking frequency ($p < 0.01$) had significant influence on the excretion time. The model explained 83% of the variance. The predicted values are listed in table 27.

Table 27: Withdrawal time (in hours) for PEN (Least Square Means (LSQ_M) and standard error) - variance factor body weight included		
Milking frequency	LSQ_M ± se	sign.*
3 times	53.5 ± 4.4	a
2 times	65.8 ± 5.1	a b
1.5 times	77.7 ± 4.9	b
* Different letters show significant differences ($p < 0.05$)		

Nafcillin

Including body weight the model still explained 91 % of variance in withdrawal time. The predicted values for nafcillin including body weight are listed in table 28.

Table 28: Withdrawal time (Least Square Means (LSQ_M) and standard error) – NAF (variance factor body weight included)		
Milking frequency	LSQ_M ± se	sign.*
3 times	31.6 ± 1.9	a
2 times	38.5 ± 2.1	b
1.5 times	47.9 ± 2.1	c
* Different letters show significant differences ($p < 0.05$)		

Dihydrostreptomycin

Including body weight the model accounted for 87 % of deviance in time. Only the milking frequency ($p < 0.01$) had a significant influence on the withdrawal time (table 29).

Table 29: Withdrawal time (Least Square Means (LSQ_M) and standard error) – DHS (variance factor body weight included)		
Milking frequency	LSQ_M ± se	sign.*
3 times	37.1 ± 3.6	a
2 times	53.1 ± 4.2	b
1.5 times	57.6 ± 4.0	b
* Different letters show significant differences ($p < 0.05$)		

4.4 Omnygram® (Colistin, Ampicillin)

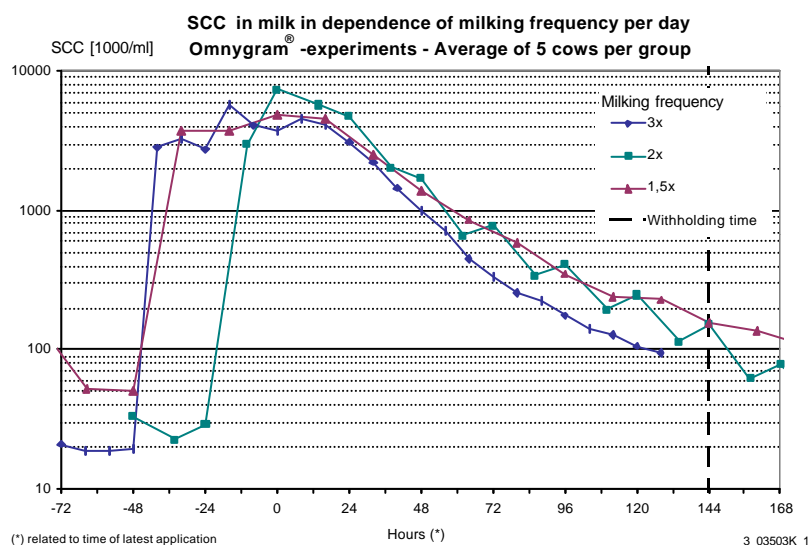
4.4.1 Status of animals

Table 30: Omnygram®-experiments – Status of animals						
Cow	No. of lactation	Days after calving	Body weight [kg]	Milk yield per day [kg]*	SCC in composite milk [1000/ml]**	Mastitis pathogens
Milking frequency 3 times per day						
1796	4	186	690	27.6	14	n.d.
1828	3	137	720	30.6	8	n.d.
1892	1	199	655	31.8	32	n.d.
1900	1	201	640	28.8	23	n.d.
1912	1	79	578	35.1	22	n.d.
Milking frequency 2 times per day						
1818	4	181	793	31.0	13	n.d.
1867	2	126	700	27.6	51	LF: CNS
1871	2	119	644	23.8	8	n.d.
1889	1	169	707	23.0	59	RH: Coryneform
1915	1	105	625	25.4	10	n.d.
Milking frequency 1.5 times per day						
1827	3	197	740	22.5	77	n.d.
1833	3	275	800	23.1	177	n.d.
1835	3	132	780	24.7	25	n.d.
1888	1	168	680	27.7	66	n.d.
1896	1	105	693	25.1	48	n.d.

*average during experimental period; ** average of anamnestic period; n.d. = not detected, CNS = coagulase-neg. staphylococci; quarters: RF = right front, LF = left front, RH = right hind, LH = left hind

4.4.2 SCC

Figure 15



In figure 15 the average SCC of cow composite milk is shown for the three trials.

4.4.3 Excretion

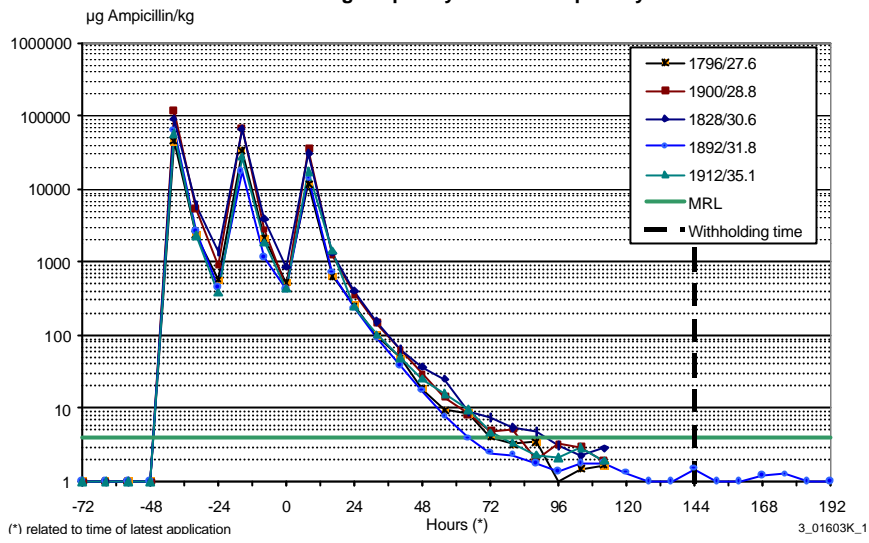
In table 31 the excreted quantities of AMP and colistin via milk with respect to the milking interval are summarized per cow and group:

Table 31: AMP and colistin excretion via milk in dependence of milking interval					
Cow no.	Milk yield (kg)	Excreted AMP		Excreted colistin	
		mg	in % of applied*	mg	in % of applied*
Milking frequency: 3 times per day					
1796	27.6	812	7.8	480	48.5
1828	30.6	1 983	19.1	922	93.1
1892	31.8	943	9.1	520	52.5
1900	28.8	2 232	21.5	938	94.7
1912	35.1	1 133	10.9	402	40.6
Average	30.8	1 421	13.7	652	65.9
Milking frequency: 2 times per day					
1818	31.0	1 871	18.0	728	73.5
1867	27.6	1 348	13.0	663	67.0
1871	23.8	1 472	14.2	856	86.5
1889	23.0	1 238	11.9	454	45.9
1915	25.4	905	8.7	556	56.2
Average	26.2	1 367	13.2	652	65.9
Milking frequency: 1.5 times per day					
1827	22.5	482	4.6	759	76.7
1833	23.1	901	8.7	836	84.4
1835	24.7	108	1.0	263	26.6
1888	27.7	516	5.0	610	61.6
1896	25.1	846	8.1	761	76.9
Average	24.6	570	5.5	646	65.3
* 100 % = 10 392 mg AMP, 990 mg Colistin					

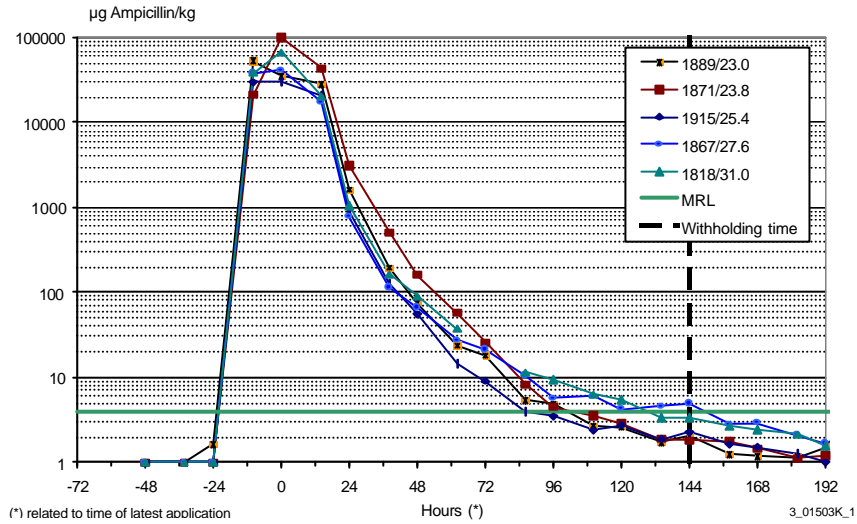
4.4.4 Withdrawal time

The excretion curves of the individual cows within each experiment and the average curves are demonstrated in figures 16.1-17.3 resp. 18.1-18.2.

**Excretion of Ampicillin (Omnygram®) in milk of individual cows
- Milking frequency three times per day**



Milking frequency two times per day



Milking frequency 1.5 times per day

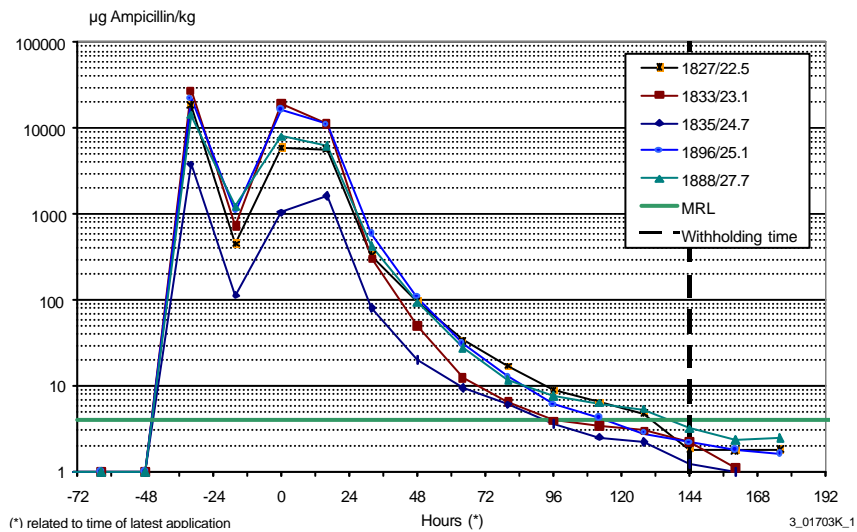
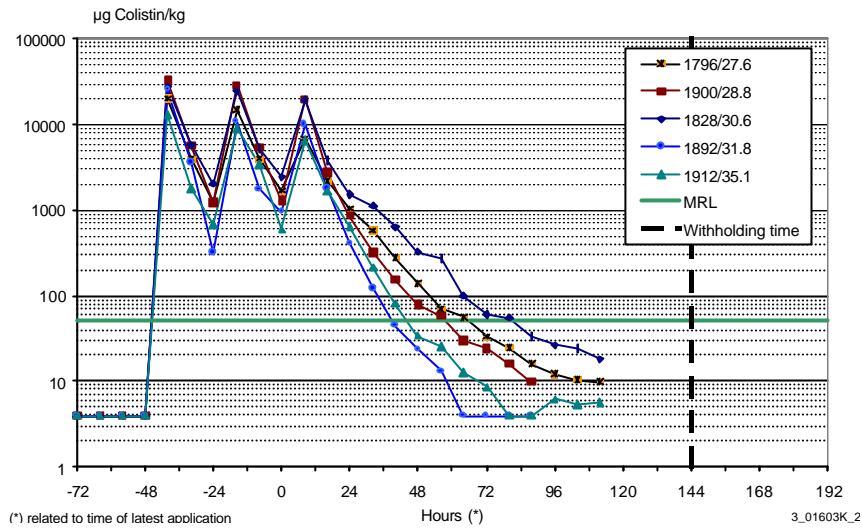
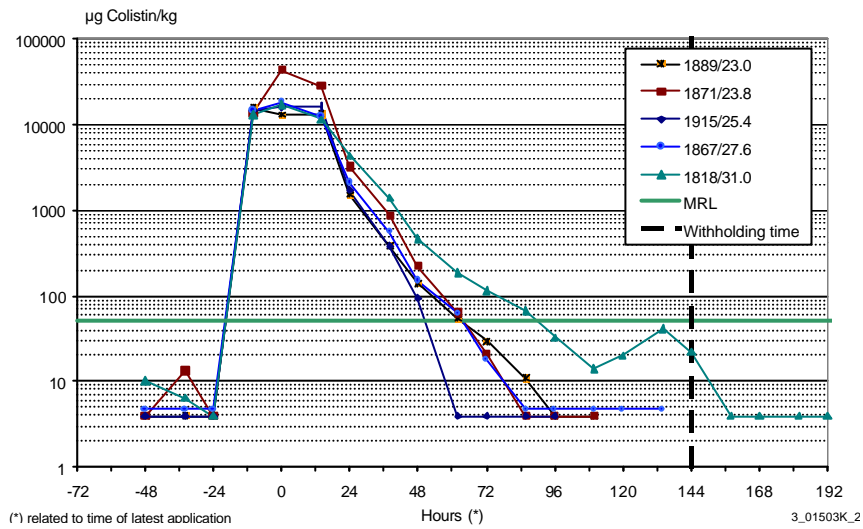


Figure 16.1-16.3

**Excretion of Colistin (Omnygram®) in milk of individual cows
- Milking frequency three times per day**



Milking frequency two times per day



Milking frequency 1.5 times per day

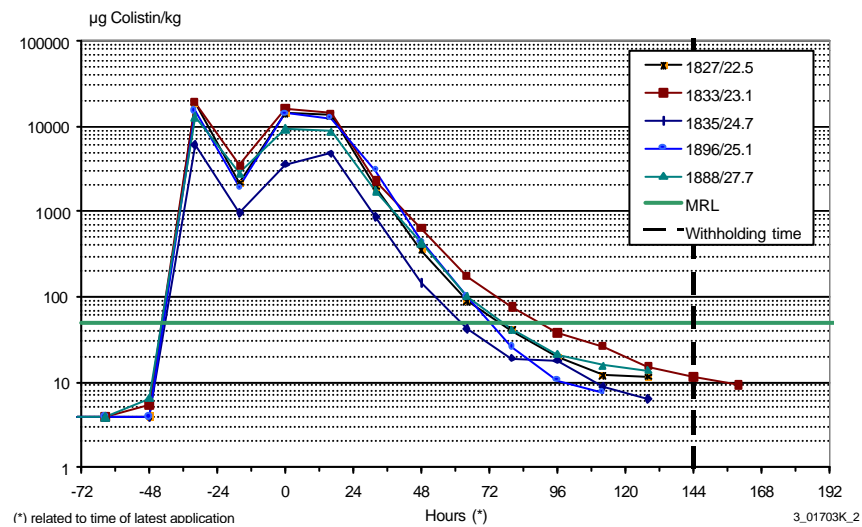
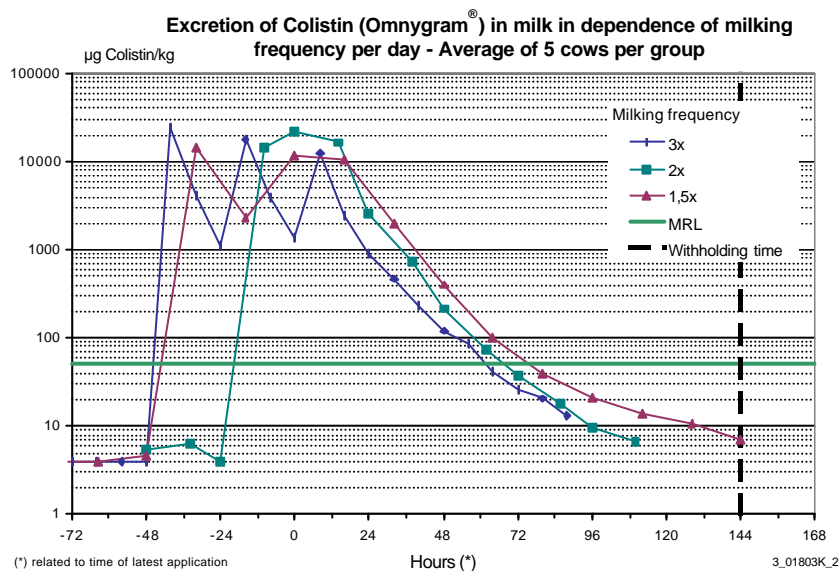
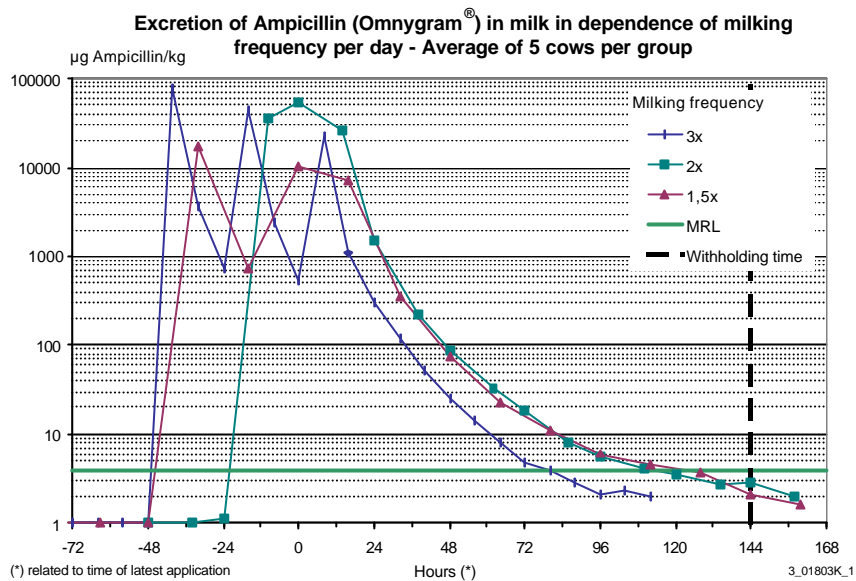
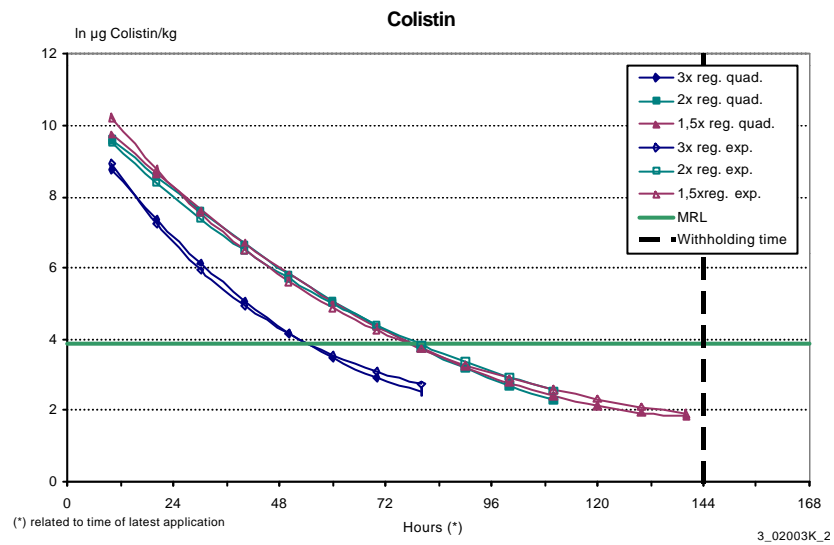
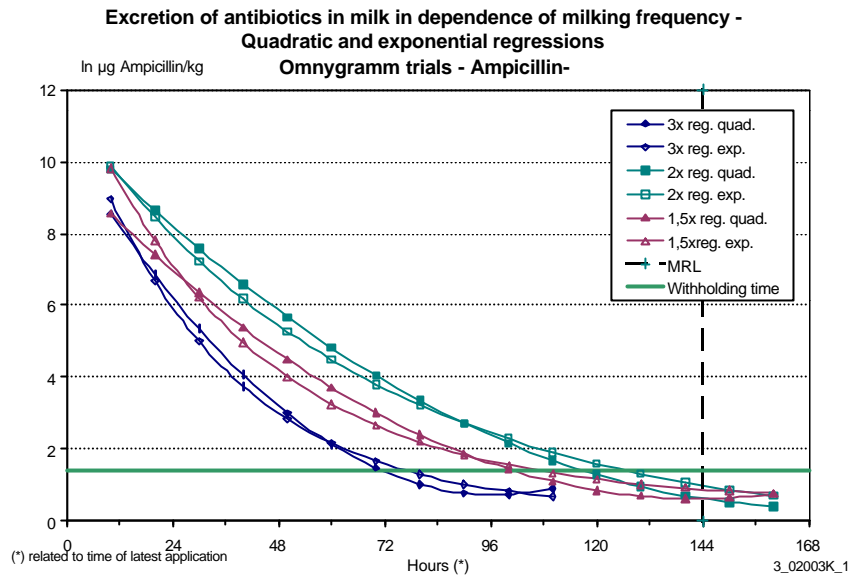


Figure 17.1-17.3



Figures 18.1-18.2

The quadratic and exponential regressions for each antibiotic are presented in figures 19.1 and 19.2.



Figures 19.1-19.2

The results of the different approaches to determine the withholding periods are summarized in table 32.

Table 32: Withdrawal time (in hours) of Omnygram® in dependence of milking frequency – summarized results from pragmatic approach, TTSC and regression model		
Official withdrawal time: 144 h		
AMP	Mean	95/95 tolerance limit/ 95% confidence interval
Milking frequency: 3 times per day		
Pragmatic – individual	78	
Pragmatic –average	76	
TTSC ^a	81	153
Quadratic regression ^b	72	*
Exponent. regression ^b	77	121
Milking frequency 2 times per day		
Pragmatic – individual	125	
Pragmatic –average	127	
TTSC ^a	127	310
Quadratic regression ^b	118	*
Exponent. regression ^b	127	193
Milking frequency: 1.5 times per day		
Pragmatic – individual	116	
Pragmatic –average	120	
TTSC ^a	123	257
Quadratic regression ^b	102	*
Exponent. regression ^b	108	*
COLISTIN	Mean	95/95 tolerance limit/ 95% confidence interval
Milking frequency: 3 times per day		
Pragmatic – individual	58	
Pragmatic –average	40	
TTSC ^a	68	219
Quadratic regression ^b	54	*
Exponent. regression ^b	54	105
Milking frequency 2 times per day		
Pragmatic – individual	81	
Pragmatic –average	79	
TTSC ^a	89	194
Quadratic regression ^b	77	124
Exponent. regression ^b	79	142
Milking frequency: 1.5 times per day		
Pragmatic – individual	72	
Pragmatic –average	72	
TTSC ^a	92	168
Quadratic regression ^b	77	97
Exponent. regression ^b	76	99
^a 95/95 tolerance limit, ^b 95 % confidence interval ; * Due to the course of the curve the 95% confidence interval cannot be derived		

4.4.5 *Analysis of variance*

The experiment with milking 2 times per day was excluded from the analysis because of deviating treatment intervals. Only milking frequencies of 3 times and 1.5 times per day were compared.

Without variance factor body weight

Ampicillin

The model accounted for 68 % of the deviance of time. None of the variables had a significant influence on the withdrawal time. The predicted values are listed in table 33.

Table 33: Withdrawal time (in hours) for AMP (Least Square Means (LSQ_M) and standard error)		
Milking frequency	LSQ_M± se	sign.*
3 times	82.9 ± 27.8	a
1.5 times	123.5 ± 18.5	a
* Different letters show significant differences (p<0.05)		

Colistin

No factor showed a significant influence on the withdrawal time. There were no significant differences between the milking frequencies. The model explained 56% of variance. The predicted values are listed in table 34.

Tab.34 : Withdrawal time (in hours) for Colistin (Least Square Means (LSQ_M) and standard error)		
Milking frequency	LSQ_M± se	sign.*
3 times	62.3 ± 20.3	a
1.5 times	69.5 ± 13.5	a
* Different letters show significant differences (p<0.05)		

Inclusive the factor body weight

Including the factor body weight into the model only slight changes in the predicted values for the withholding time were produced by this continuous variable (data not shown). None of the factors had significant influence on the withdrawal time.

5. Discussion

5.1 Influence of milking frequency on excretion of antibiotics in milk

In all experiments milking frequency showed an influence on the excretion time of antibiotics in milk leading to shorter excretion periods with increasing milking frequency. Significant differences between certain experimental groups were observed for all antibiotic compounds except ampicillin and colistin. Only for nafcillin the excretion was significantly different for all three milking frequencies. For penicillin (tested in two different drugs) only the excretion period of cows milked 3 times versus 1.5 times was significantly different. For cefquinome and dihydrostreptomycin the excretion period for milking three times was significantly shorter compared to the other two groups, but there was no significant difference between milking 2 and 1.5 times.

These results are consistent with findings from Henschelchen and Walser (1983) who observed shorter excretion periods in cows milked every two hours compared to cows milked two times per day after intramammary treatment with penicillin G respectively oxytetracycline. In these studies the applied amount was of minor importance for the duration of excretion.

For colistin there was a large variation in the excretion periods of individual cows. In this case a higher number of animals would be needed to show significant differences.

For most drugs the difference in excretion periods between cows milked 2 times and 1.5 times was smaller than between cows milked 2 times and 3 times per day, although the difference in time period is the same with 4 hours on average.

However, no conclusions on the efficacy of antibiotics with different milking frequencies can be drawn from these observations, because the concentrations in udder tissue are not known.

Penicillin was included in two treatment trials, once with an injector containing only procain-penicillin and once as a combination of penicillin-Na with nafcillin and dihydrostreptomycin. In the former the total amount of penicillin applied was about 10 times the amount of penicillin in the drug combination. The drug with the higher concentration also had a longer excretion time, but this was most probably due to the sustained release of penicillin G from procain-penicillin.

Additional factors with significant influence on the excretion time were the parameters days after calving (only for cefquinome in Cobactan[®] LC experiments) and number of lactation (penicillin and DHS in Nafpenzal[®] MC -experiments). In the Nafpenzal[®] MC trials the significant influence of lactation number on withdrawal time of penicillin and dihydrostreptomycin was neutralized by including body weight in the analysis of variance which shows that the effect of lactation number and body weight are related.

The other factors included like milk yield, body weight and SCC had no influence on the time of excretion. Additional parameters were included in a study by Seymour et al. (1988) who found

that only the applied drug had significant influence on the excretion time. None of the factors route of administration, case number (in cows treated repeatedly due to clinical mastitis), number of days treated, body weight, lactation number or milk yield had significant influence. Milking frequency was not included in that study.

5.2 Withholding periods

Except of the trials with Cobactan[®] LC none of the individual cows exceeded the MRL of antibiotic residues in milk after the end of the withholding period. However, in the Cobactan[®] LC trials one cow in each group had residue concentrations at MRL level at the last milking of the withholding period or one day later. In the experiment with the mishandled injectors (see chapter 4.1) the MRL was exceeded more frequently.

When the withholding periods were calculated by different approaches and a tolerance limit resp. a confidence interval was included, the indicated withholding period was not always sufficient.

In the European Union the official method to determine withholding periods for milk after intramammary treatment is that published by EMEA (1998), the TTSC-method.

Considering the low number of 5 animals used for each treatment trial a high tolerance factor has to be applied which results in withholding periods of up to 211 hours (almost 9 days instead of 5) for cefquinome. For this reason a minimum number of 19 cows is recommended for excretion trials within procedures for drug approval.

For penicillin the withholding period of 5 days (120 hours) would only be exceeded when the 95/95 tolerance limit for the TTSC method is applied.

Interpreting the results it has to be taken into account, that the experimental design is based on worst case conditions where the drugs were administered 3 times to all quarters of the cows. In practice, the probability of exceeding the MRL is probably lower, although in clinically diseased cows additional factors may affect the excretion time. These aspects will be addressed in deliverable D12.

The withholding period calculated by the TTSC approach resulted in the longest withholding time compared to the other approaches indicating a high level of consumer protection especially when the 95/95 tolerance limit is included.

5.3 Total amount of drug excreted via milk

The total amount of drug excreted via milk largely varied between different antibiotics. For cows milked three times the percentage of antibiotic excreted via milk was in general higher than for cows milked less often. This can be interpreted in a way that more frequent milking leads to removal of antibiotics and after every milking a new balance of concentrations between tissue and

newly secreted milk is formed. The effect is not observed with colistin, a drug that is known to be of low lipid solubility and therefore not crossing the blood milk barrier easily (Ziv, 1975).

The lowest amount excreted via milk was determined for ampicillin. The milk to serum concentration ratio for ampicillin is low (Table 1) which together with high lipid solubility leads to a diffusion from milk to serum. The highest amounts excreted via milk were observed for DHS, a polar substance which is in ionized form at physiological pH and shows low penetration of biological membranes. For colistin the total percentage excreted via milk is nearly the same for all three groups (about 65 %), so drug release and removal via milk were independent from milking frequency. These findings can be explained by the physico-chemical properties of the drug resulting in limited ability to penetrate biological membranes. A higher proportion of the total amount of drug to be excreted via milk was expected due to similar properties like polymyxin B for which 90 % excretion via milk were observed by Ziv and Schultze (1982).

For cefquinome and penicillin as single substance the amount excreted via milk by cows milked 1.5 times was higher than for those milked 2 times per day. These differences are probably due to different excretion patterns in individual cows.

5.4 Tissue reactions

The intramammary application of drugs did not lead to major rises in SCC in most treatment trials. In the trials with Omnygram[®], which contains colistin, an antibiotic which is known to cause tissue irritations, cows reacted with a dramatic increase of SCC. Even 2 days after the end of the withholding period SCC was still not back to the level in the anamnestic period. In milking times following an intramammary treatment all cows showed flakes in the milk for more than one milking time and reacted with restlessness and some kicking off the milking cluster. Considering these reactions in healthy cows it should be taken into account that in cows with clinical mastitis the time until SCC goes back to physiological levels could take much longer than the indicated withholding period for milk.

5.5 Recommendations of the manufacturer of drugs

Storage

In the first experiment with Cobactan[®] LC (cefquinome) the injectors used for the second of three intramammary administrations had been stored at a temperature exceeding the recommended storage temperature by the manufacturer. This led to a markedly prolonged excretion time in treated cows (figure 2.2 and 3).

The injectors were subsequently analyzed by the manufacturer. The analysis showed noticeable changes in macroscopic aspects with changes in colour and homogeneity as well as an increased

viscosity. The cefquinome content complied with shelf-life specifications. Probably the changes in viscosity lead to retarded release of cefquinome from the formulations thus leading to the prolonged excretion period.

The results with cefquinome clearly indicated that storage conditions deviating from those recommended by the manufacturer can lead to an extended excretion period.

Treatment intervals

Within the Omnygram[®] experiments different treatment schemes were applied for the trial with milking frequency of 2 times per day (3 treatments within 24 hours) compared to milking frequencies 3 and 1.5 times per day (3 treatments within 48 hours). Different treatment intervals were due to missing recommendations by the manufacturer. The recommended intervals were given only on request (by phone). Unfortunately the two treatment trials with 3 and 1.5 times milking per day could not be repeated.

Treatment in shorter intervals with milking 2 times per day lead to a higher concentration in milk after the third treatment. This probably explains the slightly prolonged excretion time in this experiment which was unexpected. Probably with comparable treatment intervals to the other two trials the excretion curve for this experiment would lay between the curves of the two other experiments.

5.6 Sensitivity of microbial inhibitor tests

In most countries screening tests with *B. stearothermophilus* as test microorganisms are used most often for screening milk samples for residues of antibiotics in milk quality payment schemes as well as in food control examinations. This test microorganism is especially sensitive for β -lactam antibiotics whereas other compounds are not detected with comparable sensitivity. From the detection limits derived for the compounds used in this study the penicillins penicillin G, ampicillin and nafcillin can be detected with sufficient sensitivity by these tests, whereas the cephalosporine cefquinome is only detected at higher concentrations than the MRL; the detection limits of the various *B. stearothermophilus* tests applied in this study vary broadly for this substance.

As β -lactam antibiotics are most frequently applied in the therapy of mastitis several tests for the rapid detection of this group for antibiotics have been developed. These tests are frequently applied for the examinations of car tanker milk when arriving at the dairy. In this study the receptor test Beta Star was tested. Of the included β -lactams penicillin, nafcillin and cefquinome could be detected at concentrations below MRL, whereas ampicillin gave questionable results at MRL concentration. The detection limits of *B. stearothermophilus* tests for DHS as an aminoglycoside antibiotic and colistin as a polypeptide antibiotic are much higher than the MRL concentrations

(100-10 000 times the MRL). For the screening of residues of these substances in house-validated ELISA tests with sufficient sensitivity were applied.

The results of this study underline the necessity to use method combinations for screening milk for residues of antimicrobials for aspects of food safety regarding consumers' health as well as for secure technological processes in the dairies.

6. Conclusions

1. Farmers working with an automatic milking system should ensure that treated cows are milked at least twice per day in regular intervals to make sure that the MRL in milk is not exceeded after the end of the withholding period. When dairy cows are treated with antibiotics the milking frequency has an effect on the excretion of antibiotics in the milk of individual cows leading to a longer excretion period with a reduced number of milkings per day. This effect is more or less pronounced for certain antibiotic drugs.
2. In any case the indicated withholding period has to be adhered to.
3. SCC in milk of single quarters can be increased not only due to mastitis but also due to tissue reactions in response to treatment. Screening quarter milk samples for SCC in milk is recommended after the end of the withholding period before delivering milk to the bulk tank.
4. If cow-side tests are applied additionally to make sure that milk of individual cows is free of inhibitors after the end of the withholding period, farmers should be aware that screening tests do not detect all antibiotics with sufficient sensitivity. Test systems detecting the applied drug at MRL-level should be used.
5. Recommendations of the manufacturer regarding storage temperature, treatment interval and dosage should be followed closely to prevent prolonged drug excretion times in milk. Increased storage temperature lead to a significantly prolonged excretion period in milk in case of Cobactan[®] LC. For Omnygram[®] deviations from the expected drug excretion characteristics in milk were observed according to different treatment intervals.
5. In this study only the question of influence of different milking frequencies on excretion of antibiotic residues in milk was addressed. Therefore, conclusions cannot be drawn regarding the efficacy of treatment with antibiotics under these circumstances.

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Abbreviations

ADI	Acceptable Daily Intake
AMP	Ampicillin
<i>B. stearothermophilus</i>	<i>Bacillus stearothermophilus</i>
CEF	Cefquinome
COL	Colistin
DHS	Dihydrostreptomycin
H	hour(s)
I.U.	International Unit
LOD	Limit of Detection
LOQ	Limit of Quantification
MRL	Maximum Residue Limit
n.a.	not applied
NAF	Nafcillin
n.d.	not determined
NOEL	No Observable Effect Level
PEN	Penicillin
s	Standard deviation
se	Standard error
SCC	Somatic Cell Count
XQ	Arithmetic Mean